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Though acetylcholinesterase is the primary target of organophosphorus toxicants, our finding that acetylcholinesterase knockout mice are supersensitive to the lethal effects of VX, DFP, chlorpyrifos oxon, and iso-OMPA demonstrates that other important targets exist. The goal of this work is to identify non-acetylcholinesterase targets of organophosphorus toxicants. Three living mice were treated with a dose of biotinylated organophosphate that was not toxic to the mice. The biotinylated proteins were extracted by binding to avidin-sepharose, and separated by gel electrophoresis. Coomassie stained bands were digested with trypsin, and identified by their fragmentation patterns on a quadrupole time of flight mass spectrometer. It was found that albumin was labeled by the biotinylated organophosphate. Tissues from untreated mice showed no albumin by mass spectroscopy. Confirmation that albumin was labeled by the organophosphate was obtained by Western blotting where the biotinylated organophosphate was visualized with Streptavidin Alexa 680. Mouse, bovine, and human albumin reacted with biotinylated organophosphate. It is concluded that albumin is a new biomarker of organophosphorus agent exposure.

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Abbreviations

AChE	acetylcholinesterase enzyme
BChE	butyrylcholinesterase enzyme
BSA	bovine serum albumin
CPO	chlorpyrifos oxon
DFP	diisopropylfluorophosphate
DTNB	dithiobisnitrobenzoic acid; color reagent for activity assay
FP-biotin	biotinylated organophosphate where the leaving group is the fluoride ion and the marker is biotin; 10-(fluoroethoxyphos phinyl)-N-(biotinamidopentyl) decanamide
HABA	4-hydroxy azobenzene-2-carboxylic acid
iso-OMPA	tetraisopropyl pyrophosphoramide; inhibitor for BChE
OP	organophosphorus toxicant
PVDF	polyvinylidene difluoride; membrane for binding proteins
SDS	sodium dodecyl sulfate
VX	O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate; nerve agent

Introduction

The purpose of this work is to identify proteins that react with low doses of organophosphorus agents (OP). There is overwhelming evidence that acute toxicity of OP is due to inhibition of acetylcholinesterase (AChE). However, we have found that the AChE knockout mouse, which has zero AChE, is supersensitive to low doses of OP. The AChE-/- mouse dies at doses of OP that are not lethal to wild-type mice (Duysen et al., 2001). This demonstrates that non-AChE targets are involved in OP toxicity. Our goal is to identify new biological markers of exposure to organophosphorus agents.

Our strategy uses biotinylated OP to label proteins, which are then visualized with streptavidin conjugated to a fluorophore. Labeled proteins are identified by mass spectrometry.

Since some cases of Gulf War Illness may have been caused by exposure to low doses of OP, our studies may lead to an explanation for the neurologic symptoms in some of our Gulf War veterans.

Relation to Statement of Work

In this third year of the project we have made progress on Tasks 2, 4, and 6. We have completed Tasks 1, 2, and 5. Additional work is planned on Tasks 3, 4, and 6 in the coming year.

List of tasks

Task 1

Biotinylated OP will be synthesized in the laboratory of Dr. Charles Thompson at the University of Montana. 1-20 mg of biotinylated OP will be provided to Dr. Lockridge.

Task 1 has been completed. Annual report 2002.

Task 2

The toxicity of various doses of chlorpyrifos oxon, dichlorvos, diazinon-O-analog, and malathion-O-analog will be tested in mice deficient in acetylcholinesterase. The goal is to find a dose that is toxic only to AChE deficient mice (AChE +/- and AChE -/-), and is not toxic to wild-type (AChE +/+) mice.

Task 2 has been completed during the past year and is reported here.

Task 3

Human and mouse brains will be extracted and treated with biotinylated OP. The biotinylated proteins will be purified with avidin-Sepharose, and separated by gel electrophoresis. The number and size of proteins that reacted with biotinylated OP will be visualized on blots by treating with avidin conjugated to an indicator.

Task 3 has been completed for mouse brain, but not for human brain. Annual reports 2002 and 2003. Human brain will be studied in the coming year.

Task 4

The protein bands isolated in task 3 will be partially sequenced. The partial sequences will be used to search the Human and Mouse Genome Databases, for the purpose of obtaining the complete amino acid sequences and identifying the proteins.

Task 4 was completed for mouse brain in Annual Report 2003. Human brain will be studied in the coming year. Analysis of the results has led to identification of a new biomarker for OP exposure, reported here.

Task 5

The reactivity with insecticides of the new biochemical markers will be compared to the reactivity of AChE and BChE with the same insecticides. The set of proteins identified in task 3, as well as AChE and BChE, will be ranked for reactivity with chlorpyrifos oxon, dichlorvos, diazinon-O-analog, and malathion-O-analog. This will be accomplished by measuring second order rate constants for individual proteins in brain extracts.

Task 5 has been completed. Annual reports 2002 and 2003.

Task 6

The toxicological relevance of the biochemical markers identified in task 3 will be determined. Mice will be treated with the dose of insecticide determined in Task 2, that is, a dose that is not toxic to wild-type mice, but is toxic to AChE deficient mice.

Progress on Task 6 is reported here.

Task 2

The toxicity of various doses of chlorpyrifos oxon, dichlorvos, diazinon-O-analog, and malathion-O-analog will be tested in mice deficient in acetylcholinesterase. The goal is to find a dose that is toxic only to AChE deficient mice (AChE +/- and AChE -/-), and is not toxic to wild-type (AChE +/+) mice.

Task 2 has been completed during the past year and is reported here.

Toxicity of chorpyrifos oxon, dichlorvos, and malaoxon in AChE deficient mice.

Abstract. The goal was to find a dose of OP that was toxic only to AChE deficient mice (AChE +/- and AChE -/-) and was not toxic to wild-type mice. The minimal toxic doses found were 0.75 mg/kg ip chlorpyrifos oxon, and 15 mg/kg ip malaoxon. No single dose of dichlorvos was found that produced some toxicity in AChE +/- mice and was not lethal to AChE-/- mice. Therefore dichlorvos doses were 7 mg/kg ip for AChE+/+ and +/- mice, and 1 mg/kg ip for AChE-/- mice. The best discrimination between the three AChE genotypes was obtained by malaoxon at a dose of 15 mg/kg ip. This dose of malaoxon had almost no toxic effects on AChE +/+ mice, had moderate effects on AChE+/- mice, and was severely toxic to AChE-/- mice. This information will be used to identify proteins involved in OP toxicity in Task 6

Introduction

The goal of Task 2 is to obtain information that will be useful in the completion of Task 6. In Task 2 we have determined doses of OP that can be considered low. We have defined a low dose as a dose that has no acute effects in wild-type mice but is toxic to AChE-/- mice. The plan for Task 6 is to treat mice with a low dose of OP, then analyze their brains for proteins that have reacted with OP.

Materials and Methods

Materials. Chlorpyrifos Oxon was from Dow Elanco AGR203674; O-analog recertification date April 15, 1998. MW 335 g/mole. The dry compound was dissolved in 200 proof ethyl alcohol to make a 10 mg/ml stock solution, which was stored at –80°C. Just before injection into animals, the stock solution was diluted to 1 mg/ml in 10 % ethanol (2.9 mM).

Dichlorvos was from Chem Service Inc., West Chester, PA.. (PS-89) Lot 209-

130B Purity: 98% Exp 06/00, MW 221, SG 1.42 g/ml. A 1 mg/ml stock solution was prepared in 0.9% NaCl (Sigma S8776 lot 33K2413). Aliquots were stored at –80˚C.

Malathion-O-analog (also called malaoxon) was from Chem Service, Inc., West Chester, PA. MET-86C, lot 299-68A Purity 97.8%, Exp. 09/03. MW= 330.36. Malathion-O-analog is only slightly soluble in water. Therefore corn oil (Mazola, Best Food, Englewood Cliffs, NJ) was used as the diluent. A stock solution of 5.0 mg/ml was prepared in corn oil and stored at –20°C.

Mice. The Institutional Animal Care and Use Committee of the University of Nebraska Medical Center approved all procedures involving mice. Animal care was provided in accordance with the principles and procedures outlined in the National Research Council Guide for the Care and Use of Laboratory Animals. AChE -/- mice were made by gene targeting (Xie et al., 2000) at the University of Nebraska Medical Center. The animals are in strain 129Sv genetic background. The colony is maintained by breeding heterozygotes because AChE -/- mice do not breed (Duysen et al., 2002). Wild-type mice are littermates of AChE-/- mice.

The chlorpyrifos oxon treatment group used 5 adult males of each genotype AChE -/-, +/-, and +/+. Each animal received 0.75 mg/kg i.p. The average age of each group of animals was 66 days (AChE -/-), 60 days (AChE +/-), and 71 days (AChE +/+) with a 10% variation. The average weight of each group was 19.9 g (AChE -/-), 29.0 g (AChE +/-), and 30.0 g (AChE +/+) with a standard deviation of 1-10%.

The dichlorvos treatment group used 5 adult female mice of each AChE genotype. AChE+/+ and +/- mice received 7 mg/kg ip, while AChE-/- mice received 1 mg/kg ip. The average age of each group of animals was 60 days (AChE-/-), 60 days (AChE+/-), and 55 days (AChE+/+) with a 10% variation. The average weight of each group was 16.8 g (AChE-/-), 22.1 g (AChE+/-), and 20.4 g (AChE+/+) with a standard deviation of 10%.

The malaoxon treatment group used 5 adult males of each AChE genotype. Each animal received 15 mg/kg ip. The average age of each group of animals was 75 days (AChE-/-), 78 days (AChE+/-), and 81 days (AChE+/+) with up to 17% variation. The average weight of each group was 17 g (AChE-/-), 29 g (AChE+/-), and 29 g (AChE+/+) with a standard deviation of 3-18%.

Determination of Minimum Toxic Dose. Each animal was weighed, its surface body temperature measured, and a functional observational battery of tests performed prior to treatment. OP was injected intraperitoneally with a Hamilton syringe. After the toxin was administered the animals were observed for toxic signs for a period of two hours. Temperature was measured every 5 min for the first 20 minutes, and every 10 minutes thereafter up to 2 hours.

Functional Observational Battery. The tests for toxicity (McDaniel and Moser, 1993; Moser, 1995) included observation of lacrimation, salivation, posture, gait, defecation, urination, piloerection, myoclonic jerks, tremor, arousal, body temperature, changes in body weight, vocalization, palpebral closure, reaction to being handled, hydration, mobility, stereotypic behavior including retropulsion, Straub tail, writhing,

Results

Chlorpyrifos oxon

The minimum toxic dose of chlorpyrifos oxon was 0.75 mg/kg ip for AChE +/- mice (n= 5). This dose produced very few symptoms in the wild type (n=5) and relatively severe symptoms in AChE -/- mice (n=5). The results from the functional observational battery of tests are in Table 2.1.

Table 2.1 Toxic signs in mice treated with 0.75 mg/kg chlorpyrifos oxon ip. N=5 adult male mice in each group.

ID	Age days	Wt (g)	Temp maxΔ	hunched	twitch	tremor	myoc jerks	less alert	saliva tion	lacrim ation	piloer ection
heterozygote											
M2+/-	69	28.9	+1.0	yes	no	no	no	yes	no	no	yes
M3+/-	59	28.7	+0.1	yes	yes	no	no	yes	no	no	yes
M8+/-	54	26.0	+0.9	yes	yes	no	no	yes	no	no	no
M5+/-	59	32.3	+0.3	yes	yes	no	no	yes	no	no	no
M4+/-	59	28.9	+0.9	yes	no	no	no	yes	no	no	yes
Avg	60	29.0	+0.6	5/5	3/5	0/0	0/0	5/5	0/0	0/0	3/5
SD	5.5	2.2	0.4								
Wild type											
M4+/+	78	35.5	+0.9	no	no	no	no	yes	no	no	no
M3+/+	76	28.3	+0.5	no	no	no	no	no	no	no	no
M2+/+	83	29.4	+0.3	no	no	no	no	yes	no	no	no
M3+/+	58	28.6	+0.3	yes	no	no	no	yes	no	no	yes
M6+/+	59	27.3	+0.1	no	no	no	no	yes	no	no	no
Avg	71	30.0	+0.4	1/0	0/0	0/0	0/0	4/5	0/0	0/0	1/5
SD	11.5	3.3	0.3								
nullizygote											
M6-/-	67	20.8	-1.2	yes	yes	yes	yes	yes	yes	no	yes
M8-/-	63	19.8	-1.4	yes	no	yes	yes	yes	yes	no	yes
M2-/-	64	21.4	-1.6	yes	yes	yes	yes	yes	yes	no	yes
M3-/-	64	18.6	-1.6	yes	no	yes	yes	yes	yes	no	yes
M5-/-	73	18.9	-1.0	yes	yes	yes	yes	yes	yes	no	yes
Avg	66	19.9	-1.4	5/5	3/5	5/5	5/5	5/5	5/5	0/0	5/5
SD	5.5	2.2	0.4								

The maximum change in temperature (°C) over a period of 120 minutes post-dosing is reported.

Both AChE +/- and +/+ animals had slight increases in body temperature at the 0.75 mg/kg dose of chlorpyrifos oxon, while the AChE -/- animals had a significant decrease (1.4°C ± 0.4). All AChE -/- and +/- animals displayed hunched posture at some point during the treatment, while only one AChE +/+ animal had a hunched posture. Six of ten AChE -/- and +/- animals had a twitch either in their ears or on their skin at some point in the two hours following treatment. No AChE +/+ animals exhibited a twitch or lacrimation. All five AChE -/- animals developed a clonic tremor and myoclonic jerks above their innate mild tremor. No AChE +/- or +/+ animals developed

tremors or myoclonic jerks. All of the animals tested with the exception of one AChE +/+ animal became less alert, that is, they had fewer exploratory movements and they had periods of immobility.

All five AChE -/- animals had an increase in salivation ranging from mild to severe. No AChE +/- or +/+ animals had increased salivation. Lacrimation was not observed in any animal of any genotype. All the AChE -/- animals, three of five AChE +/- animals, and one AChE +/+ animal displayed piloerection. The same wild type animal that was hunched had piloerection. Two AChE +/- animals had red paws and snout. Other cholinergic symptoms noted in the AChE -/- group included retropulsion, frantic activity for a brief period immediately after dosing, partially closed eyes, mucus in the eyes, ataxic gait, splayed legs, flattened posture, severe tremor, and Straub tail.

Dichlorvos

During dose finding experiments it was noticed that AChE+/+ and +/- mice had severe symptoms after receiving 10 mg/kg dichlorvos ip, but slight to no symptoms after 5 mg/kg. A dose of 7.0 mg/kg dichlorvos was chosen as a dose that produced mild symptoms in the +/+ and +/- animals, while eliciting a temperature difference between the genotypes. Five +/+ and five +/- animals were tested. The +/- animals had a significantly larger decrease in body temperature compared to the +/+ animals (see Figure 2.1). Treatment with 7.0 and 5.0 mg/kg dichlorvos was fatal to nullizygotes, therefore five nullizygotes were tested with 1.0 mg/kg to determine cholinergic symptoms at this lower non-lethal dose.

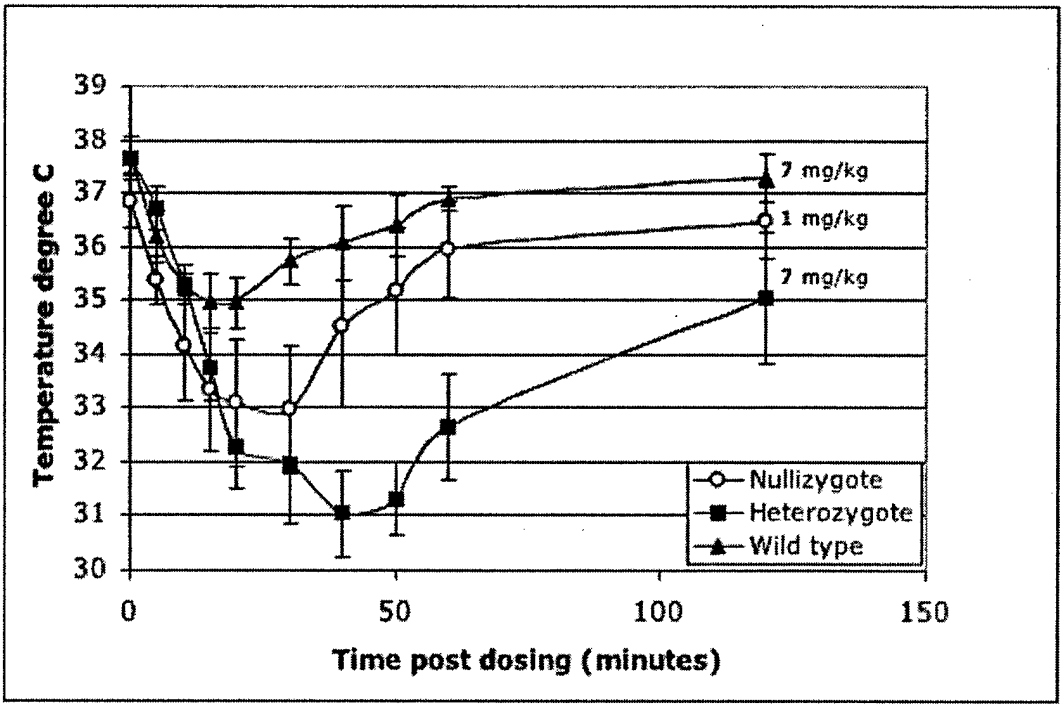

Figure 2.1. Effect of dichlorvos on body temperature. Surface body temperature was measured after ip injection of 7 mg/kg dichlorvos to AChE +/+ and +/- mice, and 1 mg/kg to AChE-/- mice (n=5 adult females per group).

Figure 2.1 shows that a dose of 7.0 mg/kg dichlorvos discriminates AChE+/+ from AChE+/- animals. Surface body temperature of AChE+/+ mice decreased 2.8°C (SD±0.5) at 15 minutes and then slowly returned to normal. In contrast the body temperature of AChE+/- animals decreased an average of 6.9°C (SD±0.8), reaching a minimum temperature 40 minutes after dosing. The temperature was still well below normal 2 hours after dosing. The body temperature of AChE-/- mice treated with 1.0 mg/kg dichlorvos decreased 4.5°C at 30 min post dosing. At two hours, the temperature was still low.

Table 2.2. Toxic signs in mice treated with dichlorvos. AChE+/- and +/+ received 7 mg/kg ip. AChE-/- received 1 mg/kg ip. N=5 adult females in each group.

ID	Age days	Wt (g)	Temp maxΔ (°)	hunched	twitch	tremor	myoc jerks	less alert	saliva tion	lacrim ation	piloer ection
Heterozygote											
F2+/-	57	18.98	-7.9	yes	yes	yes	no	yes	no	yes-mucus	yes
F2+/-	51	21.22	-5.8	yes	no	no	no	yes	no	no	yes
F3+/-	57	18.63	-7.1	yes	no	yes	no	yes	no	no	yes
F3+/-	56	22.64	-6.5	yes	no	no	no	yes	no	yes mucus	yes
F4+/-	56	20.3	-7.0	yes	no	yes	no	yes	no	no	yes
Avg	55.4	20.4	-6.9	5/5	1/5	3/5	0/5	5/5	0/0	2/5	5/5
SD	2.5	1.6	0.8								
Wild type											
F1+/+	55	22.76	-3.6	yes	no	no	no	yes	no	no	yes
F5+/+	70	23.19	-2.3	yes	no	no	no	yes	no	no	no
F9+/+	51	19.97	-2.9	yes	yes	no	no	yes	no	no	yes
F4+/+	70	24.28	-2.7	yes	yes	yes	no	yes	no	no	yes
F5+/+	56	20.5	-2.6	yes	no	yes	no	yes	no	no	yes
Avg	60.4	22.14	-2.8	5/5	2/5	2/5	0/5	5/5	0/0	0/5	4/5
SD	9.0	1.83	0.5								
Nullizygote											
F2-/-	69	19.06	-4.9	yes	yes	yes	yes	yes	no	yes	yes
F1-/-	55	15.68	-4.8	yes	yes	yes	yes	yes	no	yes	yes
F3-/-	63	16.07	-4.0	yes	yes	yes	no	yes	no	yes	yes
F3-/-	61	14.23	-4.2	yes	yes	yes	yes	yes	no	yes	yes
F5-/-	53	18.9	-4.5	yes	yes	yes	no	yes	no	yes	yes
Avg	60.2	16.8	-4.5	5/5	5/5	5/5	3/5	5/5	0/0	5/5	5/5
SD	6.4	2.1	0.4								

The maximum change in temperature (°C) over a period of 120 minutes post-dosing is reported.

The response of AChE+/+ and +/- mice to 7.0 mg/kg dichlorvos was similar in all parameters tested, with the exception of the body temperature shown in Figure 2.1. Both genotypes had hunched posture, piloerection, and were less alert. A few had twitch and tremor. None had myoclonic jerks. See Table 2.2. The cholinergic signs of toxicity were more severe in AChE-/- mice even though the dose of dichlorvos was only 1.0 mg/kg. The AChE-/- mice had hunched posture, twitch, tremor, lacrimation, piloerection, and they were less alert. A few had myoclonic jerks. In addition their rate of respiration increased, eyes were partly closed, they had splayed feet and ataxic gait, flattened body and they vocalized. No animals salivated in response to dichlorvos.

All doses of dichlorvos that caused even slight toxic symptoms in AChE +/+ and +/- animals were fatal to the -/- animals. Therefore a dose of 1.0 mg/kg dichlorvos was used in the AChE -/- animals to demonstrate cholinergic toxicity, even though this dose elicited no toxic symptoms in AChE +/+ and +/- animals.

Malaoxon

At a dose of 15 mg/kg malaoxon ip, five AChE +/- animals showed moderate cholinergic symptoms (Table 2.3) including an average drop in temperature of -2.7°C (SD±0.6), hunched posture (5/5), twitching or quivers of the ears and skin (4/5), decreased alertness (5/5), piloerection (5/5), splayed hind limbs (2/5), reduced body tone (3/5), rapid respirations (5/5), Straub tail (1/5), flattened posture (1/5), red paws and red snout (3/5). The AChE +/- mice recovered their body temperature and normal activity within 30 minutes post dosing. Wild type animals (n=5) treated with the same dose of 15 mg/kg had virtually no cholinergic symptoms. The only symptom noted was decreased arousal (4/5). The wild type animals had an average increase in temperature of 0.5°C (SD±1.0). The AChE -/- mice treated with 15 mg/kg malaoxon displayed moderately severe to severe cholinergic symptoms. These animals had an average drop in temperature of 2.5°C (SD±0.6), similar to the drop in temperature in AChE +/- animals, although the nullizygotes took longer to regain their normal body temperature. Other symptoms in AChE-/- mice included hunched posture (5/5), twitching or quivers of the ears and skin (5/5), severe whole body tremors (5/5), myoclonic jerks (5/5), decreased arousal (5/5), salivation (slight) (5/5), piloerection (5/5), splayed hind limbs (5/5), rapid respirations (5/5), flattened posture (5/5), red paws and snout (1/5), eyes partially closed (1/5), mucus in eyes (4/5), ataxic gait (5/5), penis protruding (1/5), and loose feces (2/5). Lacrimation was not found in any animal of any genotype.

Table 2.3. Toxic signs in mice treated with 15 mg/kg malaoxon ip. N=5 adult males in each group

ID	Age days	Wt (g)	Temp maxΔ	hunched	twitch	tremor	myoc jerks	less alert	saliv ation	lacrim ation	piloer ection
Heterozygote											
M6+/-	68	26.68	-3.4	yes	yes	no	no	yes	no	no	yes
M5+/-	89	29.15	-1.9	yes	no	no	no	yes	no	no	yes
M3+/-	83	27.87	-2.3	yes	yes	no	no	yes	no	no	yes
M4+/-	83	29.4	-2.7	yes	yes	no	no	yes	no	no	yes
M3+/-	69	30.4	-3.0	yes	yes	no	no	yes	no	no	yes
Avg	78	29	-2.7	5/5	4/5	0/5	0/5	5/5	0/5	0/0	5/5
SD	9.4	1.4	0.6								
Wild type											
M7+/+	88	26.88	+ 1.8	no	no	no	no	yes	no	no	no
M2+/+	92	28.86	+0.6	no	no	no	no	no	no	no	no
M5+/+	92	33.52	-1.0	no	no	no	no	yes	no	no	no
M1+/+	66	28.9	+0.8	no	no	no	no	yes	no	no	no
M3+/+	66	26.4	+0.3	no	no	no	no	yes	no	no	no
Avg	81	29	+0.5	0/5	0/5	0/5	0/5	4/5	0/5	0/0	0/5
SD	13.6	2.8	1.0								
Nullizygote											
M6-/-	71	18.64	-3.0	yes	yes	yes	yes	yes	yes	no	yes
M7-/-	71	12.97	-2.4	yes	yes	yes	yes	yes	yes	no	yes
M1-/-	92	19.05	-1.7	yes	yes	yes	yes	yes	yes	no	yes
M8-/-	71	15.77	-2.1	yes	yes	yes	yes	yes	yes	no	yes
M10-/-	71	18.03	-3.1	yes	yes	yes	yes	yes	yes	no	yes
Avg	75	17	-2.5	5/5	5/5	5/5	5/5	5/5	5/5	0/0	5/5
SD	9.4	2.5	0.6								

The maximum change in temperature (°C) over a period of 120 minutes post-dosing is reported.

A single dose of malaoxon caused very different toxicity in AChE+/+, +/-, and -/- mice. AChE+/+ mice had no symptoms, AChE+/- mice had moderate symptoms, and AChE-/- mice had severe symptoms after 15 mg/kg malaoxon ip.

Discussion

Toxic signs. Chlorpyrifos oxon, dichlorvos, and malaoxon had the same acute effects. AChE-/- mice had the most severe symptoms. These cholinesterase inhibitors produced hypothermia, tremors, gait and posture changes, decrease in exploratory behavior, and myoclonic jerks. None of the OP caused both salivation and lacrimation at the doses tested. Chlorpyrifos oxon and malaoxon caused salivation but no lacrimation, while dichlorvos caused no salivation but did cause lacrimation. This is in agreement with Moser, who had previously reported that chlorpyrifos is not as effective in producing lacrimation as other compounds (Moser, 1995). The toxic signs observed in AChE-/- mice are characteristic of cholinergic overstimulation, attributed to inhibition of AChE. However, the AChE-/- mice have no AChE enzyme or AChE protein. They do have normal amounts of butyrylcholinesterase (BChE). It seems reasonable to conclude that the mechanism of OP toxicity in AChE-/- mice involves inhibition of BChE.

Additional sites of action of OP. Toxicologists have found that OP have other biological actions in addition to their cholinesterase-inhibitory properties (Moser, 1995; Pope, 1999). Some compounds interact directly with muscarinic or nicotinic receptors. Chlorpyrifos alters the activity of the adenylyl cyclase signaling cascade (Song et al., 1997). Acylpeptide hydrolase is more sensitive than AChE to inhibition by chlorpyrifosmethyl oxon, dichlorvos, and DFP (Richards et al., 2000). The goal of this work is to identify these additional sites. The information obtained in Task 2 will aid in identifying new sites of OP action in Task 6.

Why is the minimal toxic dose different for each OP? Chlorpyrifos oxon is twenty times more potent than malaoxon in mice. If potency were explained entirely by inhibition of AChE and BChE, then potency should correlate with the rate constant for inhibition of AChE and BChE. Table 2.4 shows an 80 fold difference in the rate constants for reaction with AChE, and a 120,000 fold difference in the rate constants for reaction with BChE when comparing chlorpyrifos oxon and malaoxon.

Table 2.4. Comparison of rate constants for reaction of OP with human AChE, human BChE, and the minimal toxic dose in mice

OP	k, M^{-1} min^{-1} AChE	k, M^{-1} min^{-1} BChE	Reference	Minimal toxic dose, mg/kg ip
Chlorpyrifos oxon	1.0×10^7	1.7×10^9	(Amitai et al., 1998)	0.75
Dichlorvos	1.2×10^5	8.7×10^5	(Skrinjaric-Spoljar et al., 1973)	7 (1 for AChE-/-)
Malaoxon	1.3×10^5	1.5×10^4	(Rodriguez et al., 1997)	15

AChE-/- mice have only BChE but no AChE. Therefore, it could be expected that chlorpyrifos oxon would be 120,000 times more potent than malaoxon. This was not found. Chlorpyrifos oxon was indeed more potent than malaoxon but only 20 fold more potent. Factors other than rate of inhibition of BChE must be important. Fat solubility and ability to cross membranes probably influence toxicity. The structures of chlorpyrifos oxon and malaoxon in Figure 2.2 show both OP to be hydrophobic compounds, more soluble in organic than in aqueous solvents.

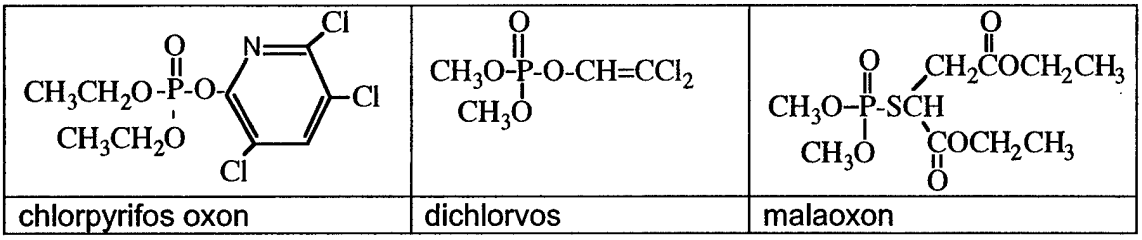

Figure 2.2. **OP structures.**

Scavengers other than BChE could play a role. In Task 4 we report that albumin is a scavenger of OP. Albumin appears to act as a sink for OP. Since the concentration of albumin is very high, exceeding the BChE concentration by 10,000 fold, a considerable amount of OP could be neutralized by binding to albumin.

Relevance to humans. Malathion is widely used. The cities and suburbs of California as well as New York city are sprayed with malathion to kill off mosquitoes that carry the West Nile virus. The active component of malathion is malaoxon. We have shown that AChE deficient mice are more senstitive to the toxicity of malaoxon than wild-type mice. By analogy, we expect that AChE deficient humans will be intoxicated by doses of malaoxon that cause no symptoms in the average person. AChE deficiency in humans has not yet been identified but it must exist. People who are sensitive to pesticides are good candidates for AChE deficiency.

Task 4

The protein bands isolated in task 3 will be partially sequenced. The partial sequences will be used to search the Human and Mouse Genome Databases, for the purpose of obtaining the complete amino acid sequences and identifying the proteins.

Progress on Task 4 is reported here.

Albumin, a new biomarker of OP exposure identified by mass spectroscopy

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Abstract. The classical laboratory tests for exposure to organophosphorus toxins (OP) are inhibition of acetylcholinesterase activity in red blood cells and inhibition of butyrylcholinesterase activity in plasma. In a search for new biomarkers of OP exposure, we treated mice with a biotinylated organophosphorus agent, FP-biotin. The biotinylated proteins were purified by binding to avidin-Sepharose, separated by gel electrophoresis, digested with trypsin, and identified from their fragmentation patterns on a quadrupole time of flight mass spectrometer. Albumin was found to be a major target of FP-biotin. Confirmation of the identity of the OP-labeled proteins was obtained by gel electrophoresis, transfer to PVDF membrane, and hybridization to Streptavidin Alexa-680. The most intense bands were 67 kDa (albumin) and 61 kDa (carboxylesterase). Biotinylated BChE gave a relatively weak band, but biotinylated AChE was not detected in plasma of FP-biotin treated mice, even though both enzymes were inhibited 50-80%. Eight additional bands of unknown identity were detected. Chlorpyrifos oxon, echothiophate, malaoxon, paraoxon, methyl paraoxon, diazoxon, diisopropylfluorophosphate, and dichlorvos competed with FP-biotin for binding to albumin. Carboxylesterase is not a biomarker in man because humans have no carboxylesterase in blood. It is concluded that albumin could serve as a new biomarker of OP exposure in man.

Introduction

Organophosphorus toxins (OP) are used in agriculture as pesticides, in medical practice as antihelminthics, in the airline industry as additives to hydraulic fluid and jet engine oil, and are stockpiled as chemical warfare agents. These compounds are known to exert their acute effects by inhibiting acetylcholinesterase (EC 3.1.1.7, AChE). The excess acetylcholine that accumulates causes an imbalance in the nervous system that can result in death (McDonough and Shih, 1997).

Religious cult members released sarin in the Tokyo subway in 1995 during the morning rush hour. 5000 people were intoxicated and 12 died. OP poisoning was correctly diagnosed based on characteristic pinpoint pupils and on laboratory tests that showed drastic inhibition of butyrylcholinesterase (EC 3.1.1.8, BChE) and AChE activities in blood. This incident illustrates the validity of measuring AChE and BChE activity in blood to confirm OP exposure. Why then do we need new biomarkers of OP exposure?

Low dose exposure is not easily diagnosed. There is wide variability in normal levels of red cell AChE and plasma BChE activities, so that a 50% decrease from the average activity could still be in the normal range. The average person has no clinical signs of poisoning after low dose exposure to OP, though a small percentage of people complains of headache, muscle weakness, and memory loss. Illnesses such as chronic fatigue, Gulf War Illness, chemical sensitivity, and air cabin crew illness have been hypothesized to be the result of exposure to low doses of OP. People who live near sites where OP stockpiles are being burned or neutralized worry about exposure. Another area of concern is the potential use of nerve agents by terrorists. In the event of an attack, people distant from the main target may receive low doses of the OP. For diagnosis of such low dose exposure, a new biomarker would be advantageous.

In this work, we used new mass spectrometry techniques to look for new biomarkers of OP exposure. Mice treated with FP-biotin, a biotin-tagged OP (Liu et al., 1999; Kidd et al., 2001), were found to have biotin-labeled albumin and carboxylesterase in their blood. These results are expected to be applicable to humans because humans also have albumin (but no carboxylesterase) in their blood.

Methods

Materials. FP-biotin was custom synthesized by Troy Voelker in the laboratory of Charles M. Thompson at the University of Montana, Missoula. Purity was checked by NMR and mass spectrometry and no evidence of contamination was detected. The molecular weight was 592.3 g/mole. FP-biotin was stored as a dry powder at –70°C. Just before use the dry powder was dissolved in 100% ethanol to a concentration of 13.3 mg/ml, and diluted with saline to 15% ethanol containing 2 mg/ml FP-biotin.

Immun-Blot PVDF membrane for protein blotting, 0.2 µm (catalog # 162-0177) and biotinylated molecular weight markers (catalog #161-0319) were from Bio-Rad

Laboratories, Hercules, CA. Streptavidin-Alexa 680 fluorophore (catalog # S-21378) was from Molecular Probes, Eugene, OR. Avidin-agarose beads (catalog # A-9207) were from Sigma-Aldrich, St. Louis, MO. Echothiophate iodide was from Wyeth-Ayerst, Rouses Point, NY. All other OP were from Chem Service Inc, West Chester, PA. Bovine Albumin Fraction V pH 5.2 (catalog # 810532 lot # 2305A) was from ICN, Irvine, CA.

Calculation of dose of FP-biotin. The dose of FP-biotin was calculated from dry weight. No correction was made for the fact that FP-biotin is a mixture of stereoisomers.

Mice. The Institutional Animal Care and Use Committee of the University of Nebraska Medical Center approved all procedures involving mice. Animal care was provided in accordance with the principles and procedures outlined in the National Research Council Guide for the Care and Use of Laboratory Animals. AChE -/- mice were made by gene targeting (Xie et al., 2000) at the University of Nebraska Medical Center. The animals are in strain 129Sv genetic background. The colony is maintained by breeding heterozygotes because AChE -/- mice do not breed (Duysen et al., 2002). Wild-type mice are littermates of AChE-/- mice.

Injection of FP-biotin into mice. Mice were injected intraperitoneally with FP-biotin dissolved in 15% ethanol to give a dose of 56, 18.8, or 5 mg/kg, or with vehicle alone. Mice were euthanized 120 min after the start of the experiment. Tissues from 6 mice were analyzed by mass spectroscopy: 2 AChE-/- FP-biotin treated, 2 AChE-/- untreated, 1 wild-type FP-biotin treated, and 1 wild-type untreated. In addition 6 wild-type mice were treated with 0, 0.5, 1.0, 5.0, 5.0, or 18.8 mg/kg FP-biotin. Blood from these latter wild-type mice was analyzed by gel electrophoresis and blotting. Activity of BChE and AChE was measured (Ellman et al., 1961).

Isolation of FP-biotin labeled protein. To prepare OP-labeled proteins for mass spectrometry, FP-biotin labeled proteins in brain and muscle were purified on avidin-agarose beads and separated by SDS polyacrylamide gel electrophoresis. Proteins from mice that had received no FP-biotin were purified by the same procedure.

Tissues were homogenized in 10 volumes of 50 mM TrisCl pH 8.0 containing 5 mM EDTA, and centrifuged to partially clarify the suspension. A detailed example of the protocol follows. The 0.96 ml of muscle homogenate (7.4 mg protein/ml) was diluted with 3.75 ml of 50 mM TrisCl pH 8.0, 5 mM EDTA to make 1.5 mg/ml protein solution. SDS was added to make the solution 0.5% SDS. The protein solution was heated for 3 min in a boiling water bath and then diluted with buffer to make the final SDS concentration 0.2%. The protein solution was incubated with 100 µl of washed avidin-agarose beads (1.9 mg avidin/ml of beads) overnight at room temperature, with continuous inversion, to bind the FP-biotin labeled proteins to the beads. Beads were washed three times with the TrisCl/EDTA buffer, containing 0.2% SDS, to remove non-specifically bound protein. Twenty-five µl of 6x SDS PAGE loading buffer (0.2 M TrisCl, pH 6.8, 10% SDS, 30% glycerol, 0.6 M dithiothreitol and 0.012% bromophenol blue) were added to the 100 µl of beads, and the mixture was heated at 85°C for 3 minutes.

This step released the biotinylated proteins from the avidin beads. Equal amounts of the bead mixture were loaded directly into two wells of a 10-20% gradient SDS PAGE (10-well format, 1.5 mm thick) and run for 4000 volt-hours in the cold room. The gel was stained with Coomassie blue G250 (Bio-Safe from BioRad), and destained with water. Coomassie G250 is reportedly 2-8 fold more sensitive than Coomassie R250 (BioRad specifications). To minimize contamination from keratin, the staining dish had been cleaned with sulfuric acid, and the water was Milli-Q purified. For the same reason, gloves were worn for all operations involving the gel.

Protein digestion protocol. The proteins separated on SDS PAGE were digested with trypsin to prepare them for identification by mass spectral analysis. The procedure we used was established in Dr. Thompson's laboratory at the University of Montana. Gloves were worn throughout these procedures, all solutions were made with Milli-Q purified water, and all glassware, plastic ware, and tools were rinsed with Milli-Q purified water to minimize keratin contamination. Each Coomassie stained band from one lane of the SDS PAGE was excised, placed into a separate 1.5 ml microfuge tube, and chopped into bits. The amount of gel excised was kept to a minimum. The gel bits were destained by washing with 200 µl of 25 mM ammonium bicarbonate (Aldrich) in 50% acetonitrile (synthesis grade, from Fisher). After three washes, the gel bits were colorless and had shrunken considerably. Residual liquid was removed and the gel bits dried by evaporation in a Speedvac (Jouan). Disulfide bonds in the protein were reduced by incubating the gel bits with 10 mM dithiothreitol (molécular biology grade, from Sigma) in 200 µl of 100 mM ammonium bicarbonate for 1 hour at 56°C. The gel pieces were then centrifuged, excess solution was removed, and the protein was alkylated with 55 mM iodoacetamide (Sigma) in 120 µl of 100 mM ammonium bicarbonate for 1 hour at room temperature in the dark. The gel bits were again centrifuged, excess solution was removed, and the bits were washed with 200 µl of 25 mM ammonium bicarbonate in 50% acetonitrile (three times). Residual liquid was again removed and the gel bits dried by evaporation in the Speedvac. The proteins were digested in the gel with trypsin, using 12.5 ng/µl of sequencing grade trypsin (Promega) in 25 mM ammonium bicarbonate. Ninety µl of the trypsin solution were added to the dry gel bits and incubated at 4°C for 20 minutes, to allow the gel to re-swell. Then 60 µl of 25 mM ammonium bicarbonate were layered over each sample and the samples were incubated at 37°C overnight (about 17 hours). Peptides were extracted by incubating each reaction mixture with 200 µl of 0.1% trifluoroacetic acid (sequencing grade from Beckman) in 60% acetonitrile for one hour at room temperature. Extraction was repeated three times and the extracts for each sample were pooled. The pooled extracts were evaporated to dryness in the Speedvac, and the dry samples were stored at -20°C until analyzed.

Mass spectral analysis. Each tryptic peptide digest was resuspended in 40 µl of 5% acetonitrile/0.05% trifluoroacetic acid/95% water. A 10-µL aliquot of the digest was injected into a CapLC (capillary liquid chromatography system from Waters Corp) using 5% acetonitrile/0.05% trifluoroacetic acid (auxiliary solvent) at a flow rate of 20 µL per minute. Peptides were concentrated on a C_{18} PepMap ™ Nano-Precolumn ™ (5 mm × 0.3 mm id, 5µm particle size) for 3 minutes, and then eluted onto a C_{18} PepMap ™

capillary column (15 cm x 75 µm id, 3 µm particle size both from LC Packings), using a flow rate of 200-300 nL per minute. Peptides were partially resolved using gradient elution. The solvents were 2% acetonitrile/0.1% formic acid (solvent A), and 90% acetonitrile/10% iso-propanol/0.2% formic acid (solvent B). The solvent gradient increased from 5% B to 50% B over 22 minutes, then to 80% B over 1 minute, and remained at 80% B for 4 minutes. The column was then flushed with 95% B for 3 minutes and equilibrated at 5% B for 3 minutes before the next sample injection.

Peptides were delivered to the Z-spray source (nano-sprayer) of a Micromass Q-TOF (tandem quadrupole/time-of-flight mass spectrometer from Waters Corp.) through a 75-µm id capillary, which connected to the CapLC column. In order to ionize the peptides, 3300 volts were applied to the capillary, 30 volts to the sample cone, and zero volts to the extraction cone. Mass spectra for the ionized peptides were acquired throughout the chromatographic run, and collision induced dissociation spectra were acquired on the most abundant peptide ions (having a charge state of 2+, 3+, or 4+). The collision induced dissociation spectrum is unique for each peptide, and is based on the amino acid sequence of that peptide. For this reason, identification of proteins using collision induced dissociation data is superior to identification by only the peptide mass fingerprint of the protein. The collision cell was pressurized with 1.5 psi ultra-pure Argon (99.999%), and collision voltages were dependent on the mass-to-charge ratio and the charge state of the parent ion. The time of flight measurements were calibrated daily using fragment ions from collision induced dissociation of [Glu^1]-fibrinopeptide B. Each sample was post-processed using this calibration and Mass Measure (Micromass). The calibration was adjusted to the exact mass of the autolytic tryptic fragment at 421.76, found in each sample.

The mass and sequence information for each detected peptide was submitted either to ProteinLynx Global Server 1.1 (a proprietary software package, from Micromass), or to MASCOT (a public access package provided by Matrix Science at http//www.matrix-science.com/cgi/index.pl?page=../home.html). Data were compared to all mammalian entries (ProteinLynx) or just mouse entries (MASCOT) in the NCBInr database (National Center for Biotechnology Information). Search criteria for ProteinLynx were set to a mass accuracy of 0.25 Da, and one missed cleavage by trypsin was allowed. Search criteria for MASCOT were set to a mass accuracy of ±0.1 Da, one missed cleavage, variable modification of methionine (oxidation) and cysteine (carbidamethylation), and peptide charge +2 and +3. Both software packages calculated a score for each identified protein based on the match between the experimental peptide mass and the theoretical peptide mass, as well as between the experimental collision induced dissociation spectra and the theoretical fragment ions from each peptide. Results were essentially the same from both packages.

Nondenaturing gel electrophoresis. 4-30% gradient polyacrylamide gels were cast in a Hoefer gel apparatus. Electrophoresis was for 5000 volt hours (200 volts for 25 hours) at 4°C.

SDS gel electrophoresis. 4-30% gradient polyacrylamide gels were cast in a Hoefer gel apparatus. Lower and upper buffers contained 0.1% SDS. Electrophoresis was for 2500 volt hours (100 volts for 25 hours) at 4°C.

Staining gels for BChE activity. Nondenaturing gels were stained for BChE activity by the method of Karnovsky and Roots (Karnovsky and Roots, 1964). The staining solution contained 180 ml of 0.2 M sodium maleate pH 6.0, 15 ml of 0.1 M sodium citrate, 30 ml of 0.03 M cupric sulfate, 30 ml of 5 mM potassium ferricyanide and 0.18 g butyrylthiocholine iodide in a total volume of 300 ml. Gels were incubated, with shaking, at room temperature for 3 to 5 hours. The reaction was stopped by washing the gels with water.

Staining gels for carboxylesterase activity and albumin. Nondenaturing gels were incubated in 100 ml of 50 mM TrisCl pH 7.4 in the presence of 50 mg beta-naphthylacetate dissolved in 1 ml ethanol, and 50 mg of solid Fast Blue RR. The naphthylacetate comes out of solution when it is added to the buffer, but enough remains in solution that the reaction works. Though the Fast Blue RR does not completely dissolve, pink to purple bands develop on the gel within minutes. A maximum of 30 minutes incubation at room temperature was needed. The gels were washed with water and photographed.

Visualizing FP-biotin labeled proteins. For determination of the number and size of proteins labeled by FP-biotin, proteins were subjected to gel electrophoresis, the proteins transferred to a PVDF membrane, and the protein bands visualized with a fluorescent probe. The details of the procedure follow.

Proteins were transferred from the polyacrylamide gel to PVDF membrane (Immun-Blot from BioRad) electrophoretically in a tank using plate electrodes (TransBlot from BioRad), at 0.5 amps, for 1 hour, in 3 L of 25 mM Tris/192 mM glycine buffer, pH 8.2, in the cold room (4°C), with stirring. The membrane was blocked with 3% gelatin (BioRad) in 20 mM TrisCl buffer, pH 7.5, containing 0.5 M NaCl for 1 hour at room temperature. The 3% gelatin solution had been prepared by heating the gelatin in buffer in a microwave oven for several seconds. The blocked membrane was washed twice with 20 mM TrisCl buffer, pH 7.5, containing 0.5 M NaCl and 0.05% Tween-20, for 20 minutes.

Biotinylated proteins were labeled with 9.5 nM Streptavidin-Alexa 680 fluorophore in 20 mM TrisCl buffer, pH 7.5, containing 0.5 M NaCl, 0.05% Tween-20 and 1% gelatin, for 2 hours, at room temperature, protected from light. Shorter reaction times resulted in less labeling. The membrane was washed twice with 20 mM TrisCl buffer, pH 7.5, containing 0.5 M NaCl and 0.05% Tween-20, and twice with 20 mM TrisCl buffer, pH 7.5, containing 0.5 M NaCl, for 20 minutes each, while protected from light.

Membranes were scanned with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) at 42 microns per pixel. The Odyssey employs an infrared laser to excite a fluorescent probe, which is attached to the target protein, and then collects the emitted light. The emitted light intensity is directly proportional to the amount of probe. Both the laser and the detector are mounted on a moving carriage positioned directly below the membrane. The membrane can be scanned in step sizes as small as 21 microns, providing resolution comparable to x-ray film. Data are collected using a 16-bit dynamic range. The fluorophore is stable in the laser, making it possible to scan the membrane repeatedly, while using different intensity settings to optimize data collection for both strong and weak signals. The membrane was kept wet during scanning.

Enzyme activity. AChE activity was measured with 1 mM acetylthiocholine after inhibiting BChE activity for 30 min with 0.1 mM iso-OMPA. BChE activity was measured with 1 mM butyrylthiocholine (Ellman et al., 1961). Carboxylesterase activity was measured with 5 mM p-nitrophenyl acetate after inhibiting AChE and BChE with 0.01 mM eserine, and after inhibiting paraoxonase with 12.5 mM EDTA.

Results

Toxicity of FP-biotin. The structure of FP-biotin is given in Figure 4.1. A dose of 56 mg/kg FP-biotin i.p. was lethal to AChE-/- mice and caused complete inhibition of BChE in plasma. A dose of 18.8 mg/kg was not lethal, but did cause severe cholinergic signs of toxicity, and inhibited plasma BChE 87%. A dose of 5 mg/kg caused only mild signs of toxicity and inhibited plasma BChE of AChE-/- mice 40%. In contrast, wild-type mice showed no signs of toxicity after treatment with 18.8 or 5 mg/kg FP-biotin i.p. even though their plasma BChE activity was inhibited to the same extent as in AChE-/- mice.

Plasma AChE activity was inhibited about 50% in wild-type mice treated with 18.8 mg/kg FP-biotin but was not noticeably inhibited by lower doses. AChE activity was not measured in AChE -/- mice because these knockout animals have no AChE activity (Xie et al., 2000). AChE has a 10-fold lower affinity for FP-biotin compared to BChE. This explains why a given dose of FP-biotin caused less inhibition of AChE than of BChE.

Plasma carboxylesterase activity was inhibited to the same extent in AChE-/- and +/+ mice. A dose of 5 mg/kg FP-biotin caused 50% inhibition while a dose of 18.8 mg/kg caused 80% inhibition.

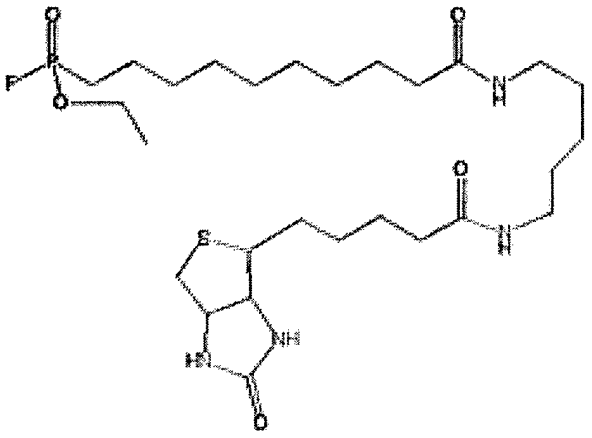

Figure 4.1. Structure of FP-biotin. The OP has a reactive phosphonofluoridate group tethered to biotin via a spacer arm.

Identification of FP-biotinylated proteins by mass spectroscopy. Brain and muscle proteins from mice that had been treated with FP-biotin, as well as from untreated control mice, were isolated by binding to avidin beads. The proteins were released from avidin by boiling in SDS and separated by SDS gel electrophoresis. Protein bands visible with Coomassie blue staining were excised and digested with trypsin. Fragmentation of tryptic peptides yielded amino acid sequence information characteristic of the protein. The proteins listed in Table 4.1 were reproducibly identified in three separate experiments. Though they were found in samples prepared from brain and muscle, they are characteristic of blood, and were probably introduced into the samples from residual blood in the tissues. Albumin was identified by 17 peptides. A representative mass spectrum of an albumin peptide is shown in Figure 4.2. These 17 peptides represented 51% of the mouse albumin sequence, leaving no doubt that albumin was labeled by FP-biotin. The untreated control tissues did not show albumin, thus demonstrating that only biotinylated albumin had bound to avidin beads. This control experiment eliminated the possibility that the albumin might have bound nonspecifically to the avidin beads.

The FP-biotinylated proteins in Table 4.1 are blood proteins. The Es1 carboxylesterase isoform is present only in mouse plasma. Albumin is the major protein in mouse plasma where its concentration is 50 mg/ml.

FP-biotinylated AChE and BChE were not found because these proteins are not abundant enough to give a Coomassie blue stained band on SDS gels. In this work only proteins that gave a Coomassie stained band were analyzed by mass spectroscopy.

Table 4.1. Mass spectral identification of blood proteins that became biotinylated after treatment of mice with FP-biotin. These proteins did not show up in untreated mice.

Protein	MW kDa	Genbank#	MOWSE score	% cover	# peptides identified
Albumin	67	Gi5915682	1157	51	17
Es1 carboxylesterase	61	Gi22135640	655	40	11

MOWSE score: **Mo**lecular **W**eight **Se**arch, a measure of the probability of a match between the experimental data and the peptide mass in the database. Scores greater than 69 are significant ($p<0.05$). % cover is the percent of the protein represented by the sequenced peptides.

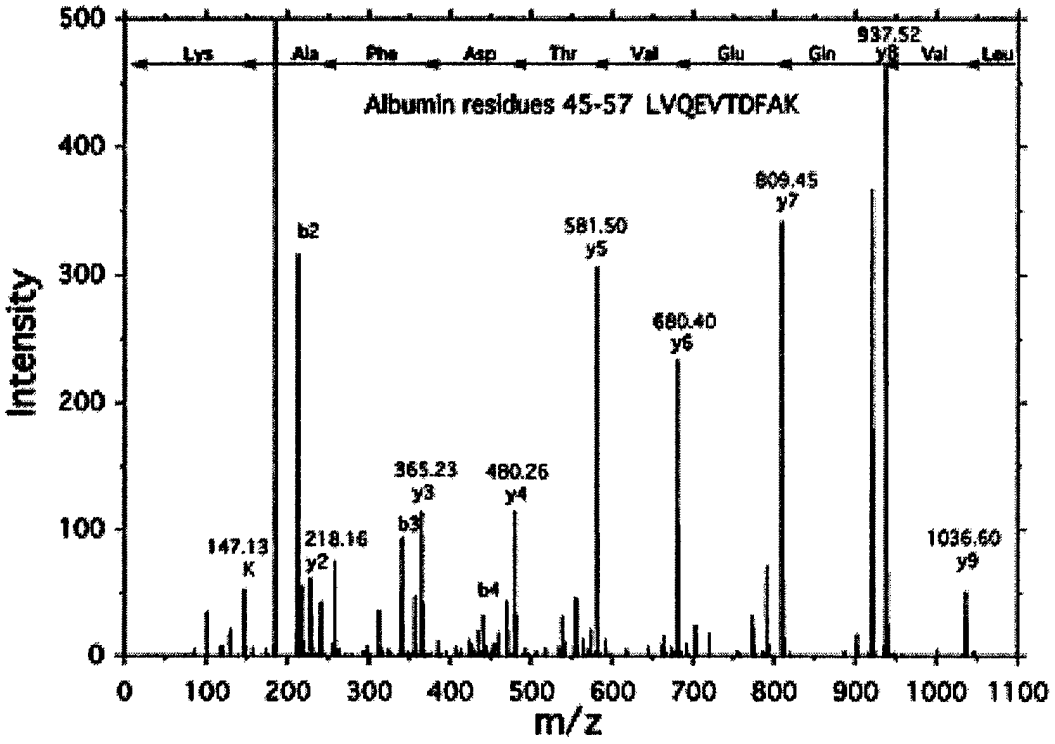

Figure 4.2. Identification of albumin by mass spectrometry. Tandem collision induced mass spectrum of peptide LVQEVTDFAK of mouse albumin. The parent ion with a single positive charge has m/z 1149.64. The FP-biotinylated albumin was recovered from 4 mice treated with FP-biotin.

Identification of endogenous biotinylated proteins. Endogenous biotinylated proteins were identified by mass spectroscopy. The proteins in Table 4.2 were found in untreated as well as in FP-biotin treated mice. They are propionyl CoA carboxylase alpha, pyruvate carboxylase, and methylcrotonyl CoA carboxylase alpha. These endogenous biotinylated proteins bound to avidin beads and were abundant enough to be visualized as Coomassie blue bands on an SDS gel.

Table 4.2. Proteins identified in untreated as well as FP-biotin treated mice. These proteins bound to avidin-agarose and were identified by mass spectroscopy

Protein	Genbank#	MOWSE score	% cover	MW kDa	# peptides identified
Propionyl CoA carboxylase alpha	Gi29612536	1309	53	80	20
Pyruvate carboxylase	Gi464506	92	7	130	2
Methylcrotonyl CoA carboxylase alpha	Gi31980706	830	46	79	15

FP-biotin does not cross the blood brain barrier. Mass spectroscopy showed no evidence of FP-biotin crossing the blood brain barrier. All the biotinylated proteins found in brain were also present in blood. FP-biotin treated AChE-/- mice showed no inhibition of BChE in brain, supporting the conclusion that FP-biotin does not cross the blood brain barrier.

Blot showing FP-biotinylated protein bands. A blot showing the proteins in plasma that became labeled with FP-biotin after treatment of mice with FP-biotin, is shown in Figure 4.3. Doses of 1 and 5 mg/kg FP-biotin were not toxic to the animals. The intense broad band in the middle of the gel resolved into three bands upon serial dilution of mouse plasma. The top band in the triplet is carboxylesterase (CE), the middle band is albumin and the bottom band has not been identified.

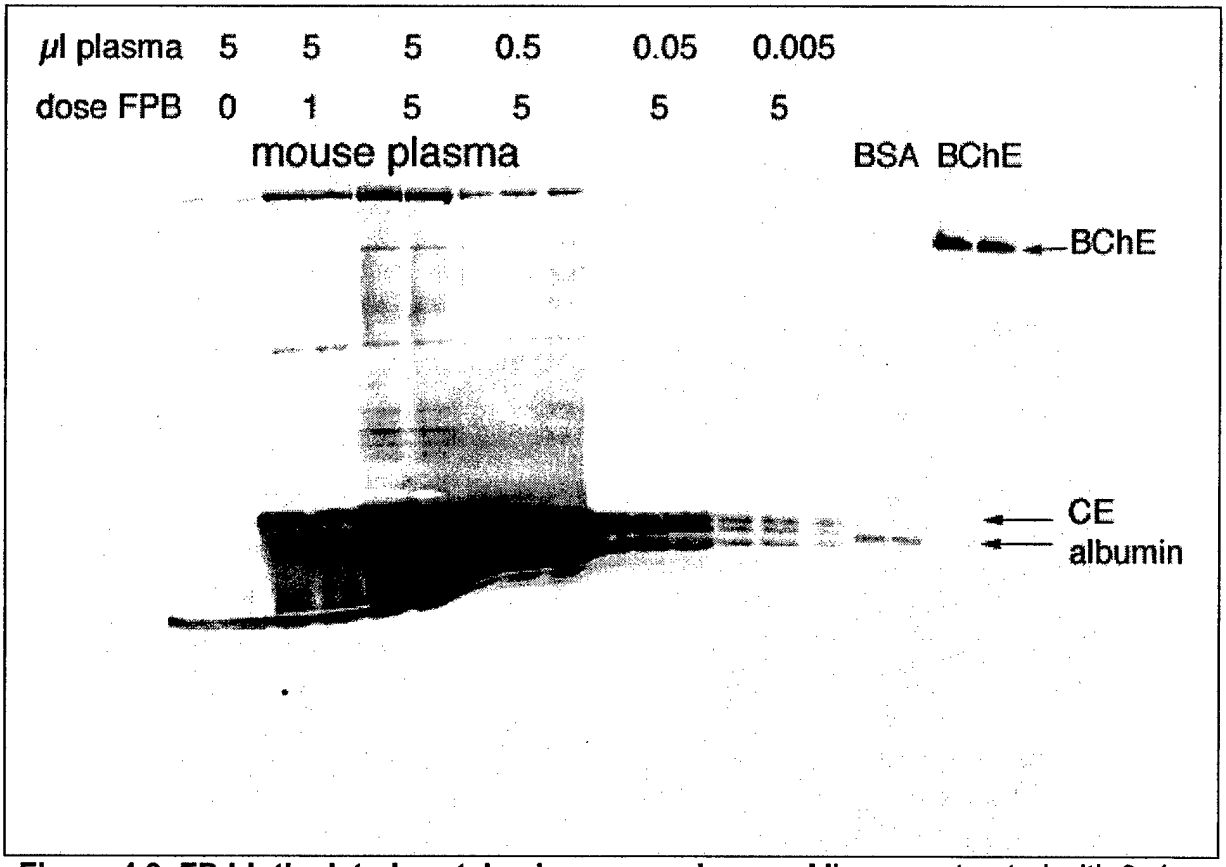

Figure 4.3. FP-biotinylated proteins in mouse plasma. Mice were treated with 0, 1, or 5 mg/kg FP-biotin ip. Two hours later blood was collected. Plasma proteins were separated on a nondenaturing 4-30% gradient polyacrylamide gel. Proteins were transferred to PVDF membrane and biotinylated proteins were visualized by hybridizing the blot with Streptavidin Alexa-680. The volume of mouse plasma loaded per lane ranged from 5 to 0.005 µl. The biotinylated bovine serum albumin standard was prepared by incubating 10 µM BSA with 20 µM FP-biotin, and loading 0.25 µg BSA per lane. The biotinylated human BChE standard was prepared by incubating 50 nM BChE (3 units/ml) with 10 µM FP-biotin, and loading 0.020 µg BChE per lane.

A band for biotinylated BChE was visible in plasma from mice treated with 5 mg/kg but not 1 mg/kg FP-biotin. This is consistent with our finding that BChE activity was inhibited 35% after treatment with 5 mg/kg but was not inhibited after treatment with 1 mg/kg FP-biotin.

In addition to albumin, carboxylesterase, and butyrylcholinesterase, mouse plasma contains about 8 other biotinylated bands whose protein identity is unknown. The intensity of the band at the top of the gel is higher than that of mouse BChE, suggesting that this protein is more abundant than BChE and that it is highly reactive. The intensity of the other bands is equal to that of BChE or lower. Thus, mouse plasma contains at least 11 proteins that bind OP at physiological conditions, at doses of OP that produce no toxic signs.

Less than 1% of the albumin in mouse plasma is estimated to have bound the FP-biotin. However, there is so much more albumin (50 mg/ml) than BChE (0.003 mg/ml) in mouse plasma that albumin consumes a significant amount of OP. Albumin scavenges 10 times more of a dose of OP than is scavenged by endogenous BChE.

Activity stained gels. The gels for Figures 4.3–4.5 were nondenaturing polyacrylamide gels. Nondenaturing gels were used because under these conditions the BChE tetramer of 340 kDa separated well from the 66 kDa albumin. This separation is not possible on SDS gels because the BChE monomer of 85,000 MW overlaps with the 67,000 MW albumin. The albumin is 10,000 times more abundant in plasma than BChE and therefore spreads into a broad band on SDS gels that overlaps with BChE and makes it impossible to visualize BChE in plasma. A second reason for using nondenaturing gels is that nondenaturing gels allow identification of BChE and carboxylesterase based on activity. Figure 4.4 shows activity with beta-naphthylacetate. The intense band is carboxylesterase (EC 3.1.1.1, CE) in mouse plasma. The bubble below carboxylesterase is albumin. BChE as well as several unidentified proteins also react with beta-naphthylacetate. Figure 4.5 shows activity of blood proteins with butyrylthiocholine. The BChE tetramer is the intense band near the top of the gel. By aligning bands in Figures 4.3, 4.4, and 4.5 we confirmed the identities of biotinylated albumin, carboxylesterase, and BChE in Figure 4.3.

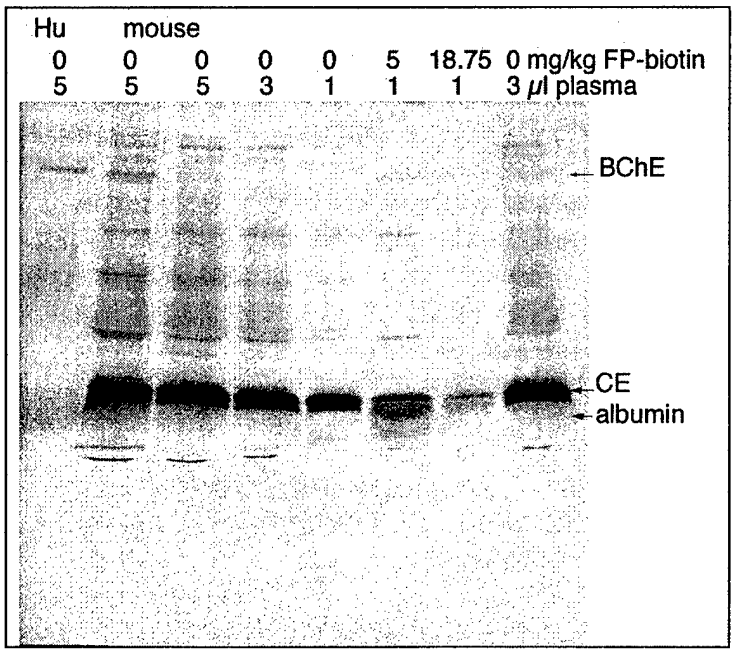

Figure 4.4. **Nondenaturing gel stained for carboxylesterase activity**. Mice were treated with 0, 5, or 18.75 mg/kg FP-biotin ip. Blood was collected two hours later. 1-5 µl of plasma was loaded per lane. The intense band is carboxylesterase (CE). Albumin migrates immediately below carboxylesterase. Note that human plasma contains no carboxylesterase.

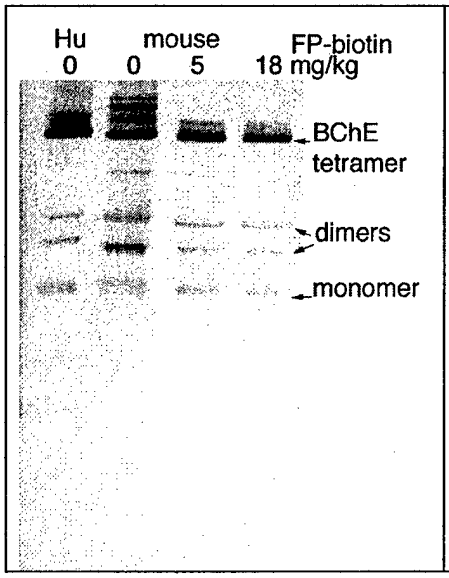

Figure 4.5. **Nondenaturing gel stained for BChE activity.** Mice were treated with 0, 5, or 18.75 mg/kg FP-biotin ip. Blood was collected two hours later. 5 µl plasma was loaded per lane. Despite 35 to 80% inhibition of mouse BChE, enough BChE activity remained to give the characteristic pattern of BChE bands. The tetramer band contains about 95% of total activity. Monomer and dimer bands are minor components. Note that the human and mouse BChE tetramer bands migrate to similar positions.

Other OP compete with FP-biotin for binding to albumin. The goal of this experiment was to determine whether other OP bind to albumin. We wanted to know whether binding to albumin was a special property of FP-biotin or whether other OP also bound to albumin. If the OP binding site is a specific tyrosine (Murachi, 1963; Black et al., 1999) then pretreatment with other OP was expected to block binding of FP-biotin. Human plasma was used in this experiment because it does not contain carboxylesterase. The absence of carboxylesterase facilitated interpretation of the results as there was no confusion between carboxylesterase and albumin. Figure 4.6 shows that other OP competed with FP-biotin for binding to albumin to varying extents. Pretreatment with chlorpyrifos oxon, echothiophate, malaoxon, paraoxon, methyl paraoxon, diazoxon, dichlorvos, and DFP reduced the binding of FP-biotin to human albumin. The only OP tested that gave no evidence of binding to albumin was iso-OMPA.

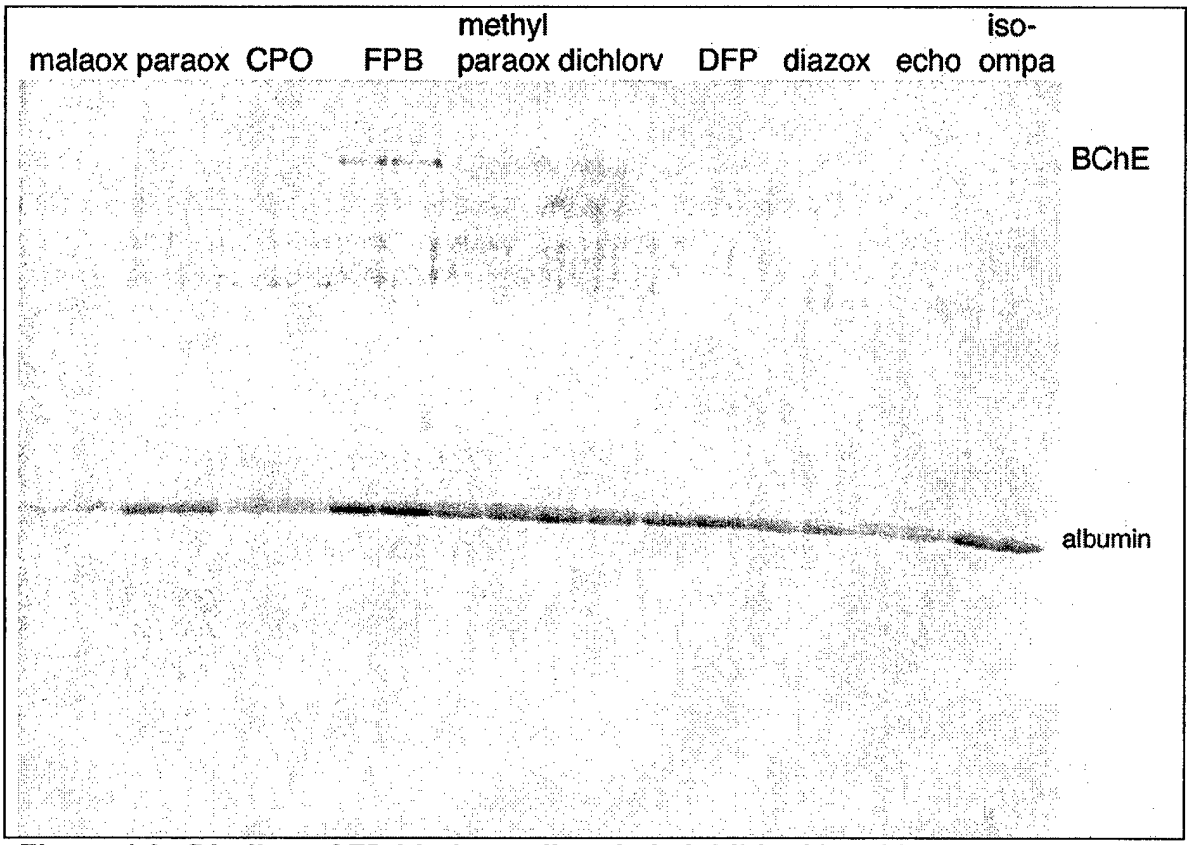

Figure 4.6. Binding of FP-biotin to albumin is inhibited by chlorpyrifos oxon and other OP. Human plasma was diluted 1:100 to reduce the albumin concentration to 10 µM. The diluted plasma was reacted with 10 mM OP for 1 h in 20 mM TrisCl pH 7.5 at 25°C. Then FP-biotin was added to 10 µM and allowed to react for 1 hour. 10 µl containing the equivalent of 0.1 µl plasma was loaded per lane, in duplicate, on a nondenaturing gel. Biotinylated proteins were visualized with Streptavidin Alexa-680 after transfer to PVDF membrane. The OP were malaoxon, paraoxon, chlorpyrifos oxon (CPO), FP-biotin (FPB), methyl paraoxon, dichlorvos, diisopropylfluorophosphate (DFP), diazoxon, echothiophate, and iso-OMPA.

These results suggest that OP binding to albumin is a general property of OP. It is to be noted that in this report OP have been demonstrated to bind to albumin from three species: mouse, bovine, and human.

Pretreatment with albumin does not protect from OP toxicity. Wild-type mice, strain CD1, received 0.2 ml of a 250 mg/ml bovine albumin solution iv. Five minutes later they were treated with a maximum tolerated dose of echothiophate subcutaneously. This 0.11 mg/kg dose of echothiophate was not lethal but it did produce cholinergic signs of toxicity. A slightly higher dose, 0.12 mg/kg, was lethal and therefore was not used. Five mice were treated only with 0.11 mg/kg echothiophate and 5 mice were pretreated with 50 mg albumin followed by 0.11 mg/kg echothiophate. Blood was withdrawn at 15 min, 1 h, 4 h, 24 h, and 48 h for assay of AChE and BChE activity in plasma. Mice pretreated with albumin had the same toxic signs and the same percent inhibition of plasma AChE and BChE activity as mice that received no albumin. It was concluded that pretreatment with this dose of albumin did not protect mice from OP toxicity.

Discussion

OP labels proteins that have no active site serine. Organophosphorus toxins are well known as inhibitors of serine proteases and serine esterases. Enzymes such as trypsin, chymotrypsin, thrombin, AChE, BChE, acyl peptide hydrolase, and carboxylesterase have a conserved active site serine with the consensus sequence GXSXG. When the active site serine is alkylated by OP, the enzyme loses activity. Loss of enzyme activity allows one to conveniently measure reactivity with OP.

Proteins with no catalytic activity are a novel class of OP target proteins. They have no active site serine and were not expected to bind OP in living animals. The advent of quadrupole time of flight mass spectroscopy has made it possible to positively identify albumin as a protein that binds OP in living mice.

Experiments with purified proteins have documented covalent attachment of OP not only to serine, but also to tyrosine and histidine. For example, human albumin covalently binds sarin and soman at tyrosine (Black et al., 1999). Papain binds DFP on tyrosine (Chaiken and Smith, 1969), while rabbit liver carboxylesterase binds DFP on histidine as well as on the active site serine (Korza and Ozols, 1988). Bromelain is not inhibited by DFP but it does react with DFP leading to the formation of a fully active, phosphorus-containing enzyme (Murachi, 1963; Murachi et al., 1965). Bovine serum albumin is readily phosphorylated by DFP with a stoichiometry of 1 DFP molecule bound per molecule of albumin (Murachi, 1963).

Living animals have previously been demonstrated to bind OP to noncholinesterase sites. The tissue distribution of radioactive DFP in rabbits was reported to have no correlation with cholinesterase activity (Jandorf and McNamara, 1950). Traub administered radiolabeled soman to rats and concluded that the distribution of radiolabel did not correlate with cholinesterase localization (Traub, 1985). Similarly, there was no correlation between the disposition of radioactive soman and cholinesterase inhibition in mouse brain (Little et

al., 1988). The disposition of radiolabeled soman changes with time: kidney, heart, nasal region, lung, skin, striated muscle, lacrymal glands, and salivary glands have high radioactivity 5 min after exposure. After 2 hours the label was found in the urinary bladder, intestinal lumen, and gallbladder, as well as in the earlier sites. After 24 hours, labeling was reduced in most organs but was still high in lung and skin (Kadar et al., 1985). These results have been interpreted to mean that tissue proteins other than cholinesterase are capable of binding OP poisons (Jandorf and McNamara, 1950; Kadar et al., 1985).

Application to diagnosis of OP exposure. Albumin is the most abundant protein in plasma. It reacts covalently with FP-biotin. However, FP-biotin is not an OP to which humans are likely to be exposed because this is a custom synthesized OP made especially for this project. Still, using FP-biotin we have demonstrated that other OP bind to albumin. Thus, our results are applicable to other OP. This means that analysis of blood for the presence of OP labeled albumin could provide a new measure for the diagnosis of OP exposure.

Existing mass spectrometric methods for diagnosis of OP exposure.
Gas chromatography combined with mass spectroscopy was used to detect sarin in archived blood samples from victims of the 1995 Tokyo subway attack (Polhuijs et al., 1997). The covalently bound sarin in 0.12 to 0.5 ml of serum was released by incubation with 2 M potassium fluoride at pH 4.0. This method confirmed that the people had been exposed to OP and, in addition, identified the OP as sarin. The mass to charge ratios of the released OP fragments were 81, 99, and 125, values characteristic of sarin.

The GC-MS method is a significant advance over simple inhibition assays, but it has limitations. 1) The method relies on being able to release the OP from its covalent attachment site on BChE with potassium fluoride. The release step requires the catalytic machinery of BChE to be intact. Samples that have been stored in ways that denature the BChE would not be amenable to this analysis. 2) Proteins other than the cholinesterases may not be able to release OP upon treatment with potassium fluoride. 3) OP-derivatized protein samples that have lost an alkyl group from the phosphonate in the process called "aging" would not be capable of releasing their OP upon treatment with potassium fluoride. 4) The method assumes that BChE is the only protein in human plasma that covalently binds OP.

GC-MS is also used to detect OP metabolites in blood and urine (Shih et al., 1991; Nakajima et al., 1998; Hui and Minami, 2000). GC-MS of OP metabolites is the method recommended by the Centers for Disease Control for monitoring potential exposure to nerve agents. Measurement of OP metabolites is limited by the fact that these compounds are rapidly cleared from the body. Sarin metabolites were found in urine on post-exposure days 1 and 3, and in trace amounts on day 7 in a man who had inhaled a dose that made him unconscious (Nakajima et al., 1998). Urine samples of 4 patients hospitalized for sarin exposure showed that most of the sarin metabolites had cleared within 24 hours (Hui and Minami, 2000).

Many of these limitations were overcome in an approach where plasma BChE was purified by affinity chromatography and digested with pepsin. The peptides were separated by liquid chromatography, and the OP-derivatized peptide as well as the OP it carried were

identified by electrospray tandem mass spectrometry (Fidder et al., 2002). This method is well-suited to detect OP exposure, and will become even more valuable if OP-derivatized proteins other than BChE are included in the analysis. A major advantage of using protein-bound OP to diagnose exposure is that the OP-derivatized proteins remain in the circulation for weeks.

What makes albumin an attractive biomarker for OP poisoning? Albumin binds more OP than is bound by BChE and albumin survives in the circulation longer than BChE. The half-life of albumin in plasma is about 20 days in humans (Chaudhury et al., 2003). This is nearly twice as long as the half-life of 10-14 days for BChE in humans (Cohen and Warringa, 1954; Jenkins et al., 1967; Ostergaard et al., 1988). Labeling BChE with DFP does not affect its half-life in the circulation of humans (Cohen and Warringa, 1954). The long half-life of OP-labeled proteins contrasts with the short half-life of OP and OP metabolites in urine. This provides a significant advantage to a method that uses OP-labeled proteins for diagnosis of OP exposure.

Task 6

The toxicological relevance of the biochemical markers identified in task 3 will be determined. Mice will be treated with the dose of insecticide determined in Task 2, that is, a dose that is not toxic to wild-type mice, but is toxic to AChE deficient mice.

Relation to statement of work: The following work was essential to understanding why AChE and BChE were not showing up in the mass spectrum of OP treated mouse tissues.

Reaction kinetics of biotinylated organophosphorus toxicant, FP-biotin, with human acetylcholinesterase and human butyrylcholinesterase

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Abstract. A biotinylated organophosphate could be useful for identifying proteins that react with organophosphorus toxicants (OP). FP-biotin, 10-(fluoroethoxyphosphinyl)-N-(biotinamidopentyl)decanamide, was synthesized and found to be stable in methanol and chloroform, but less stable in water. Since acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) are known to be sensitive targets of OP, their reactivity with FP-biotin was tested. The rate constant for reaction with human acetylcholinesterase was 1.8×10^7 $M^{-1}min^{-1}$, and for human butyrylcholinesterase it was 1.6×10^8 $M^{-1}min^{-1}$. A phosphorus stereoisomer, constituting about 60% of the FP-biotin preparation, appeared to be the reactive species. The binding affinity was estimated to be > 85 nM for AChE and > 5.8 nM for BChE. It was concluded that FP-biotin is a potent OP, well suited for searching for new biomarkers of OP exposure.

Introduction

There is overwhelming evidence that acute toxicity of OP is due to inhibition of AChE. However, the AChE knockout mouse, which has zero AChE, is supersensitive to low doses of OP. The AChE -/- mouse dies at doses of OP that are not lethal to wild-type mice (Xie et al., 2000; Duysen et al., 2001). This demonstrates that non-AChE targets are involved in OP toxicity. New biological markers of exposure to organophosphorus agents could potentially be identified by labeling proteins with FP-biotin, separating the labeled proteins on avidin-agarose, and identifying the biotinylated proteins by mass spectrometry.

To validate this strategy the reactivity of FP-biotin with AChE and BChE must be evaluated. FP-biotin has a larger structure than most organophosphorus pesticides and

nerve agents. Does this large molecule fit into the long, narrow active site gorge of AChE (Sussman et al., 1991) and BChE (Nicolet et al., 2003)? Rat tissues treated with FP-biotin have allowed identification of 7 FP-biotinylated proteins by mass spectroscopy, but none of these was AChE or BChE (Liu et al., 1999; Kidd et al., 2001).

Methods

Synthesis of FP-biotin. Troy Voelker, in Dr. Thompson's laboratory, synthesized, and purified 15.5 mg of 10-(fluoroethoxyphosphinyl)-N-(biotinamidopentyl) decanamide (FP-biotin). See Figure 6.1 for the structure of FP-biotin. The steps in the synthesis of FP-biotin followed the procedure of (Liu et al., 1999). The overall yield for the multistep synthesis was 6%. FP-biotin was purified by washing the crystals sequentially with diethyl ether and ethyl acetate. The identity of the biotinylated OP and of the intermediates was confirmed by ^{1}H, ^{13}C and ^{31}P NMR, absorbance spectra in the UV-Vis wavelength range, and combustion analysis. Phosphorus NMR in chloroform showed a doublet (36.0 and 29.3 ppm from H_3PO_4) a result consistent with P-F coupling. The doublet is the result of fluorine splitting with J = 1069 Hz. Mass spectrometry (TOF MS ES+) showed that FP-biotin had a mass to charge ratio of 593.5 (*m/z*). The expected *m/z* for FP-biotin, $C_{27}FH_{50}N_4O_5PS + H^+$, is 593.3. There was a peak at 297.3 (m/z), i.e. 1/2 of the major peak, and at 615.6 (m/z), i.e. the major peak plus one sodium. No evidence for contamination was detected.

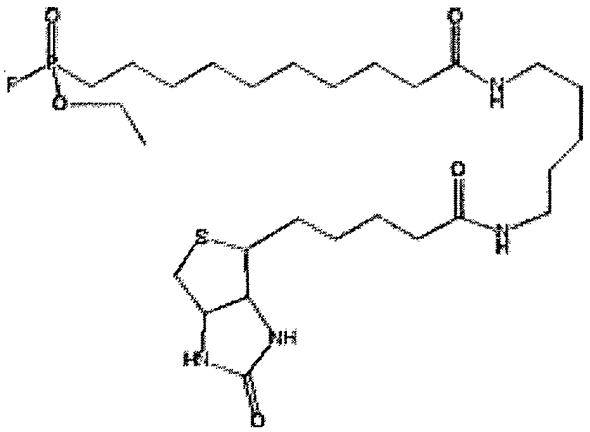

Figure 6.1. FP-biotin structure. The OP has a reactive phosphonofluoridate group tethered to biotin via a spacer arm. The phosphorus is a stereoisomeric center.

Storage of FP-biotin. Lyophilized FP-biotin was stored dry, in quantities of 3-8 mg, at -20°C, in sealed glass ampoules. Prior to use, a portion was dissolved in a small volume of solvent, (chloroform from Fisher, HPLC grade; or methanol from either EM Science, HPLC grade, or Sequemat, sequencing grade), divided into aliquots, and dried under vacuum in a Speedvac. The aliquots were in Pyrex tubes that had been cleaned in a muffle oven. The dried aliquots were returned to storage at -20°C. Though high quality solvents were used to make the aliquots, those prepared in methanol became partly degraded. Fluorine was lost from the phosphorus, as indicated by the

appearance of a new singlet in the phosphorus NMR. Aliquots re-dissolved in methanol (and not dried) remained stable for months at –20°C, suggesting that degradation occurred during vacuum drying and not during storage in methanol. When aliquots were made in chloroform, no degradation was found.

AChE and BChE. Wild-type human AChE was secreted by CHO K1 cells into serum free culture medium and purified by affinity chromatography on procainamide-Sepharose. Wild-type human BChE was purified from human plasma by ion exchange chromatography at pH 4.0, followed by affinity chromatography on procainamide-Sepharose, and ion exchange chromatography at pH 7.5 (Lockridge, 1990).

Rate constant for inhibition of human AChE by FP-biotin. The kinetics for inhibition of purified human AChE by FP-biotin were studied in 0.1 M potassium phosphate buffer pH 7.0 at 25°C. Bovine serum albumin (BSA), normally included to stabilize AChE, was omitted because it reduced the apparent first-order inhibition constant measured with FP-biotin. Inhibition of AChE was initiated by mixing 0.17 nM of highly purified human AChE with various amounts of FP-biotin, in 2 ml of potassium phosphate buffer. The FP-biotin stock solution was in 10% methanol, 90% water. At defined times a 10 µl aliquot was assayed for residual AChE activity with 1.0 mM acetylthiocholine, and 0.5 mM dithiobis-2-nitrobenzoic acid (DTNB) in 0.1 M potassium phosphate buffer pH 7.0, at 25°C (Ellman et al., 1961).

Rate constant for inhibition of human BChE by FP-biotin. Kinetics for the inhibition of human BChE by FP-biotin were studied in 0.1 M potassium phosphate buffer pH 7.0 at 25°C. Wild type human BChE (0.35 nM) was incubated with various amounts of FP-biotin for defined times, in 2 ml of potassium phosphate buffer. Inhibition was stopped by diluting a 10 µl aliquot 300 fold into a mixture containing 1 mM butyrylthiocholine and 0.5 mM DTNB for determination of residual BChE activity (Ellman et al., 1961).

Rate constants for diazoxon. The second-order rate constants for inhibition of human AChE and BChE by diazoxon were determined as described for FP-biotin. Diazoxon was from Chem Service, Inc. (West Chester, PA). Diazoxon was dissolved in methanol and stored at –70°C. Working solutions were in pH 7 buffer. Diazoxon was unstable in water, but stable in buffer. Overnight incubation in water at room temperature resulted in complete hydrolysis of diazoxon. In contrast, when diazoxon was incubated in buffer at pH 7.1 the diazoxon was still 30% intact after 5 days at room temperature.

Western blot. FP-biotin-inhibited-BChE and AChE were applied to an SDS polyacrylamide gel. After electrophoreses the proteins were transferred to PVDF membrane. To detect the biotin, the membrane was treated with an avidin-horseradish peroxidase conjugate (BioRad), and then with a chemiluminescence reagent (LumiGLO from Kirkegaard & Perry) to visualize horseradish peroxidase activity. Emitted light was detected using x-ray film.

Substoichiometric reaction of FP-biotin with BChE. FP-biotin (0.11-0.78 µM)

dissolved in water was mixed with BChE (0.61 µM), in a total volume of 100 µl of 0.1 M potassium phosphate buffer, pH 7.0 at 25°C. Inhibition reached completion during the mixing time, and the remaining activity was stable for at least an hour. The amount of active BChE remaining after inhibition was determined by adding a 10 µl portion of the reaction mixture to 3 ml of 0.1 M potassium phosphate buffer, pH 7.0 containing 0.5 mM DTNB and 1 mM butyrylthiocholine, at 25°C. Hydrolysis of the butyrylthiocholine was followed by the absorbance increase at 412 nm (Ellman et al., 1961). The concentration of BChE in the reaction mixture was calculated from the activity of an uninhibited sample, where 720 units/ml is 1 mg/ml of BChE, or 11.7 nmoles per ml. A unit of activity is defined as µmoles butyrylthiocholine hydrolyzed per min. The concentration of FP-biotin was determined by a combination of biotin titration and ^{31}P-NMR.

Concentration of FP-biotin. The total concentration of biotin in the FP-biotin preparation was determined by titration against an avidin/4-hydroxy azobenzene-2-carboxylic acid complex as described by Green (Green, 1965). The biotin concentration measured both the intact FP-biotin and its hydrolysis product. Both 4-hydroxy azobenzene-2-carboxylic acid (HABA) and avidin were from Sigma. The fraction of the preparation containing intact FP-biotin was determined by ^{31}P-NMR. The total concentration of biotin and the non-hydrolyzed fraction of FP-biotin were used to calculate the concentration of intact FP-biotin.

^{31}P-NMR. ^{31}P-NMR data, for samples containing 120-130 µM FP-biotin (total concentration of hydrolyzed and non-hydrolyzed), were collected on a 500 MHz spectrometer (Varian INOVA), over 16 hours, at 25°C. Signals were proton decoupled. The repetition rate was 5 seconds. Peak positions were measured relative to 85% phosphoric acid at 0.0 ppm. Linear prediction was used in all experiments to flatten the baseline and improve signal quality. Data were collected by Paul Keifer at the NMR Core Facility at the University of Nebraska Medical Center.

Inhibition potency of the other isomer of FP-biotin. The assumption in this experiment was that FP-biotin was a mixture of stereoisomers and that one isomer preferentially reacted with BChE. We wanted to know whether the other isomer could react with trypsin or with other proteins. An FP-biotin solution was depleted of the BChE-reactive isomer. Then trypsin was added and trypsin activity was tested with time. The substrates for trypsin were Nα-p-toluene-sulfonyl-L-arginine methyl ester HCl and Nα-benzoyl-DL-arginine-p-nitroanilide (Sigma).

In a second approach, 5.1 nmoles of FP-biotin were depleted of BChE-reactive isomer by reaction with 5.1 nmoles of BChE. After 1 h the mixture was spun through a 30,000 MW cutoff filter to separate residual FP-biotin from FP-biotin bound to BChE. The residual FP-biotin was reacted with mouse brain homogenate for 5 h. The positive control was the reaction of FP-biotin with mouse brain homogenate. Samples were boiled in SDS gel loading buffer and loaded on a 10-20% polyacrylamide SDS gel, at 30 µg of mouse brain protein per lane. After electrophoresis and transfer of proteins to PVDF membrane, the biotin was visualized by reaction with StreptAvidin Alexa 680 (Molecular Probes, Eugene, OR). Fluorescence emission at 700 nm was recorded on an Odyssey flat bed scanner (Li-COR, Lincoln, NE).

The amount of FP-biotin in the 30,000 MW cutoff filtrate was quantified with avidin/HABA to be sure that the expected amount was actually present.

Results

Concentration of FP-biotin. The concentration of FP-biotin as determined by titration with avidin and HABA (Green, 1965) was the same as the concentration calculated by weight for the lyophilized compound. This meant that the dry compound did not contain salts. The titration with avidin and HABA detected total biotin and did not distinguish between intact FP-biotin and FP-biotin that had lost its fluorine. ^{31}P-NMR was used to measure the amount of defluorinated FP-biotin.

FP-biotin dissolved in chloroform was completely intact as indicated by peaks at 35.2 and 29.9 ppm with a fluorine splitting of 1070 Hz (Figure 6.2). No third peak appeared during the 16-hour data acquisition period. In contrast, FP-biotin dissolved in water showed a doublet at 40.1 and 34.8 ppm (with a fluorine splitting of 1070 Hz) and a singlet at 29.9 ppm (Figure 6.2). The singlet was taken to be the de-fluoro hydrolysis product of FP-biotin. The amount of singlet was consistent with the amount of hydrolysis expected to occur in the 16-hour, data collection period.

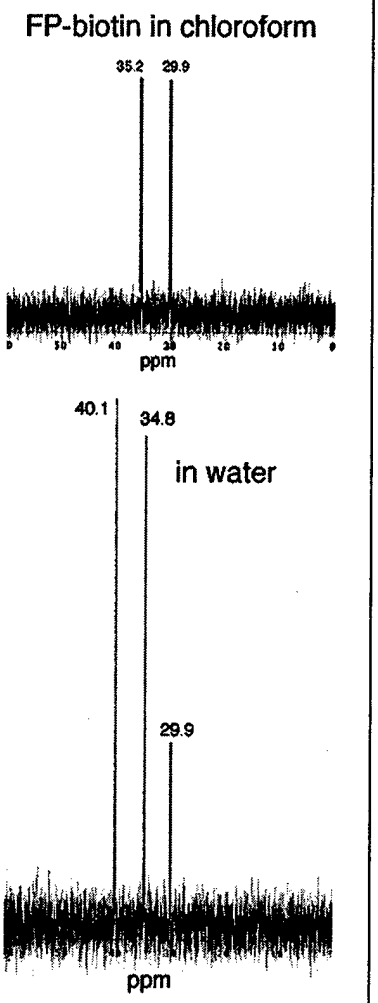

Figure 6.2. ^{31}P-NMR of FP-biotin dissolved in chloroform and in water. Data collection was for 16 h at 25°C. The FP-biotin in chloroform remained intact but the FP-biotin in water degraded about 13%.

Stability of FP-biotin in water. The stability of FP-biotin in water was assessed by its ability to inhibit BChE activity. Aliquots of stock FP-biotin (0.26 mM in methanol) were diluted 1000-fold into water to yield 0.26 µM test solutions of FP-biotin at zero time. Test solutions were stored at room temperature in capped vials, either in Pyrex tubes or colorless, plastic microfuge tubes. The apparent first-order rate constant for FP-biotin inhibition of BChE was determined by incubating 20 µl aliquots of an FP-biotin test solution with BChE (in 1.87 ml of 0.1 M potassium phosphate buffer pH 7.0, at 25°C) for defined times (Figure 6.3 inset). Active BChE remaining at the end of each incubation was determined according to the method of Ellman et al., (Ellman et al., 1961). Semi-log plots of inhibition time versus residual activity were linear for at least 90% of the reaction, from which apparent first-order rate constants for FP-biotin inhibition could be determined (Figure 6.3 inset).

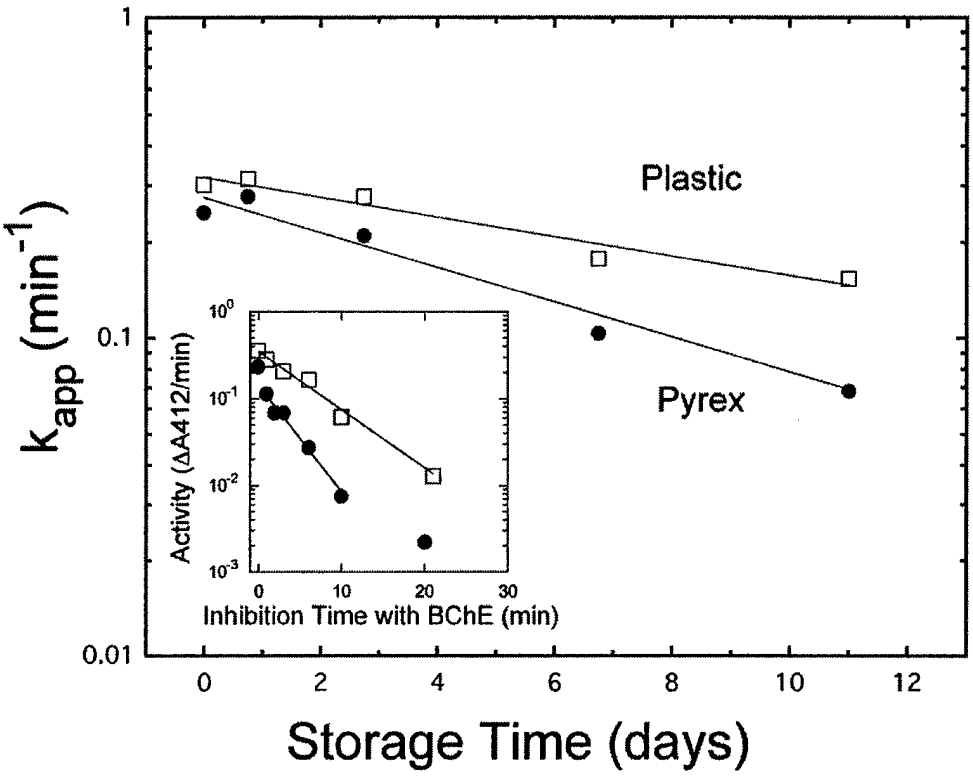

Figure 6.3. Decay of FP-biotin in water. Storage time is the time FP-biotin was left at room temperature; k_{app} is the apparent first-order rate constant for the inhibition of BChE by FP-biotin. Squares show the results for FP-biotin stored in a plastic microfuge tube, circles for storage in a Pyrex tube. Lines are from fits to a first-order process.

Inset: The inhibition of BChE by FP-biotin on day-0 (circles) and day-11 (squares), by FP-biotin dissolved in water and stored in a plastic microfuge tube. The x-axis is the time in minutes that BChE was incubated with FP-biotin before BChE activity was measured. The y-axis is the log of activity measured as change in absorbance at 412 nm per minute. Lines are from fits to a first-order process. Data were fit using SigmaPlot (v 4.16 Jandel). The slope of the line yields k_{app} per min.

The inhibition potency of the FP-biotin test solution in water decreased with time of storage at room temperature in a first-order fashion. A plot of the apparent first-order rate constant for FP-biotin inhibition of BChE turnover versus the storage time for the FP-biotin test solution gave a half-life for loss of FP-biotin of 5.5±0.4 days (in glass) or 9.9±1.5 days (in plastic) (Figure 6.3). Aqueous stock solutions of FP-biotin are stored routinely in colorless, plastic microfuge tubes from Midwest Scientific.

FP-biotin is unstable in buffer. A 170 μM FP-biotin solution was completely degraded by 20 h incubation in 0.1 M potassium phosphate pH 7.0 at 25°C. The fluoride ion was released from FP-biotin and a new peak representing de-fluorinated FP-biotin appeared in the ^{31}P-NMR spectrum. Complete loss of inhibition potency was confirmed by the failure of this FP-biotin solution to inhibit BChE.

60% of the FP-biotin reacts. The fraction of FP-biotin capable of inhibiting BChE was

determined by substoichiometric reaction with BChE, as described in Methods. BChE activity remaining after substoichiometric reaction with FP-biotin was linearly dependent on FP-biotin concentration (Figure 6.4). However, the apparent concentration of FP-biotin required to fully inhibit the BChE was greater than the BChE concentration, indicating that only a fraction of the total, intact FP-biotin was capable of inhibiting BChE. In two separate experiments, 55% and 65% of the intact FP-biotin was capable of inhibiting BChE.

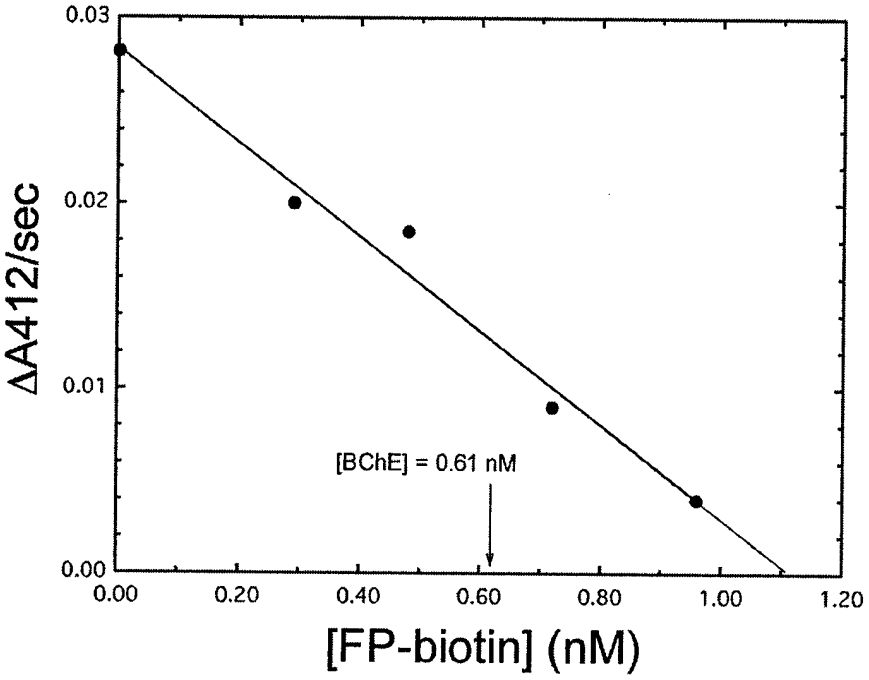

Figure 6.4. Substoichiometric titration of BChE with FP-biotin. Points are the data; the line was fit to a linear equation. This titration shows that 1.1 μM FP-biotin was required to completely inhibit 0.61 μM BChE. An arrow at 0.61 μM FP-biotin demonstrates that an intercept at 0.61 μM FP-biotin would not fit the data.

This suggested that a portion of FP-biotin was effectively unreactive toward BChE. The possibility that the unreactive FP-biotin represented degraded material was ruled out by NMR.

To test whether intact FP-biotin was responsible for the inhibition of BChE, the inhibited BChE was checked for the presence of covalently attached biotin. It was reasoned that an inhibitory contaminant in the FP-biotin preparation either would be missing the biotin moiety or would be incapable of forming a covalent adduct with BChE. SDS gel electrophoresis was used to test covalent association of biotin to protein. FP-biotin-inhibited human BChE and FP-biotin-inhibited human AChE were subjected to electrophoresis on an SDS polyacrylamide gel. The proteins labeled with biotin were detected with avidin conjugated to horse radish peroxidase (Figure 6.5). Since the gel was run in SDS, the biotin found migrating with BChE must have been covalently attached.

Therefore, inhibition did not appear to be due to a contaminant.

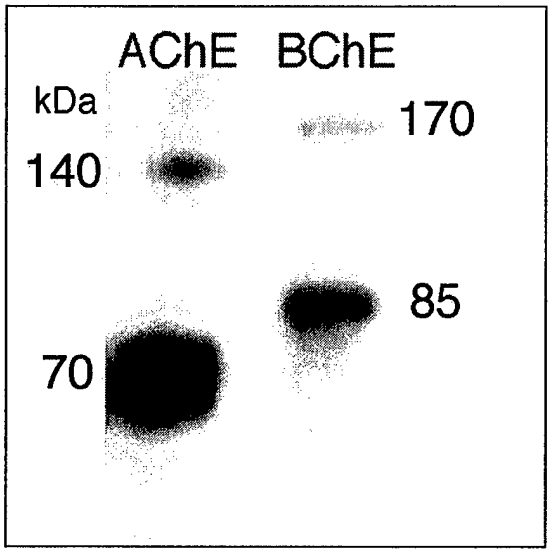

Figure 6.5. Blot of FP-biotin-inhibited-AChE and BChE. 2 to 5 pmoles of protein from an SDS gel were transferred to PVDF membrane. The blot was developed with horseradish peroxidase conjugated to avidin. This confirms the identity of the inhibitor as FP-biotin (rather than a contaminant) because the inhibitor was covalently attached and carried a biotin tag.

40% of the FP-biotin is unreactive. The question in this experiment was whether the fraction of FP-biotin that did not react with BChE, was nevertheless capable of reacting with other proteins. FP-biotin was depleted of the BChE-reactive fraction by incubation with BChE for 1 h. The residual FP-biotin was separated from BChE and incubated with mouse brain homogenate. Figure 6.6 shows that residual FP-biotin did not label the proteins in mouse brain to a significant extent. The two intense bands at 75 and 140 kDa are endogenous biotinylated proteins. In contrast, FP-biotin that had not been precleared with BChE, labeled about 50 proteins in mouse brain. It was concluded that only one stereoisomer of FP-biotin reacted with proteins in mouse brain and that this same stereoisomer reacted with human BChE.

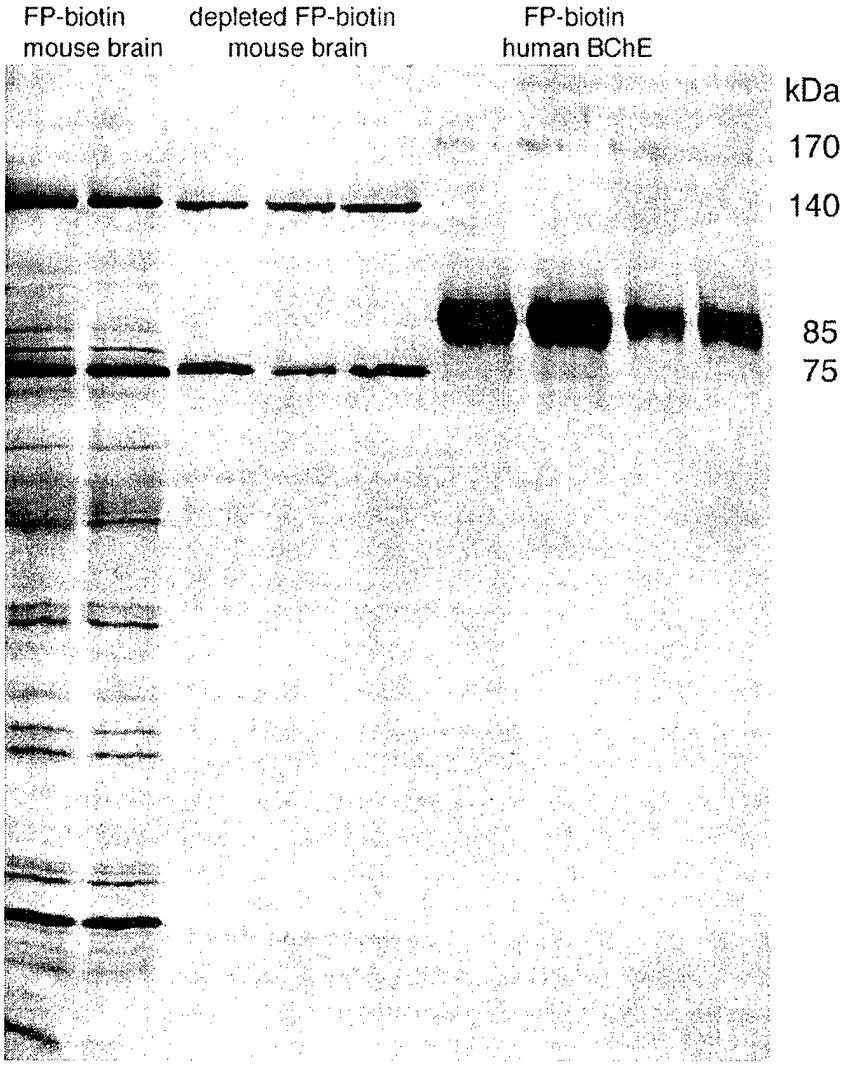

Figure 6.6. Only one stereoisomer of FP-biotin labels proteins in mouse brain. Lanes 1 & 2, mouse brain homogenate incubated with a mixture of FP-biotin stereoisomers, 30 µg protein per lane. Lane 3, 4, & 5, mouse brain homogenate incubated with residual FP-biotin, after the FP-biotin had been depleted of the BChE-reactive stereoisomer. The bands at 140 and 75 kDa are endogenous biotinylated proteins. Lanes 6,7, 8 & 9, FP-biotin labeled human BChE 1 and 0.3 pmole per lane. 1 pmole BChE = 85 ng.

The trypsin catalytic triad is a mirror image of that in AChE and BChE (Sussman et al., 1991). The question we asked was whether a stereoisomer of FP-biotin that did not react with BChE, could react with trypsin. When trypsin was treated with an equimolar amount of FP-biotin, about 50% of the trypsin was inhibited. In contrast, when FP-biotin was depleted of the BChE-reactive fraction, the residual FP-biotin did not inhibit trypsin. Experiments where BChE and trypsin were mixed confirmed an observation previously

reported by Darvesh et al (Darvesh et al., 2001) that BChE enhanced the activity of trypsin. The trypsin experiments led to the conclusion that only one stereoisomer of FP-biotin was capable of inhibiting trypsin and that this was the same stereoisomer that inhibited BChE.

Reaction kinetics of purified human AChE with FP-biotin. Inhibition of human AChE (0.17 nM) by FP-biotin was first-order for at least 90% of the reaction at all concentrations of FP-biotin tested (see Figure 6.7, inset for selected examples). A plot of the apparent first-order rate constant for inhibition versus the concentration of FP-biotin was linear from 4.3 to 43 nM FP-biotin (Figure 6.7). The concentration of FP-biotin was corrected for losses due to handling, and for 60% inhibition potency. The second-order rate constant for the reaction of human AChE with FP-biotin was determined from this linear portion of the graph to be $1.8 \pm 0.02 \times 10^7$ $M^{-1}min^{-1}$.

Extrapolation of the second-order line to zero FP-biotin revealed a small non-zero y-axis intercept, which is consistent with a minor, spontaneous loss of AChE activity due to the absence of BSA.

The dissociation constant of FP-biotin for human AChE was estimated to be greater than 85 nM. Figure 6.7 shows that the data points for FP-biotin concentrations 56 nM and higher fell below the extrapolated second-order line, suggesting the onset of saturation and the existence of a non-covalent complex between FP-biotin and AChE. The reaction of FP-biotin with AChE was too fast to allow use of FP-biotin concentrations greater than 85 nM. This meant that the dissociation constant could not be measured more precisely than the approximate value of 85 nM.

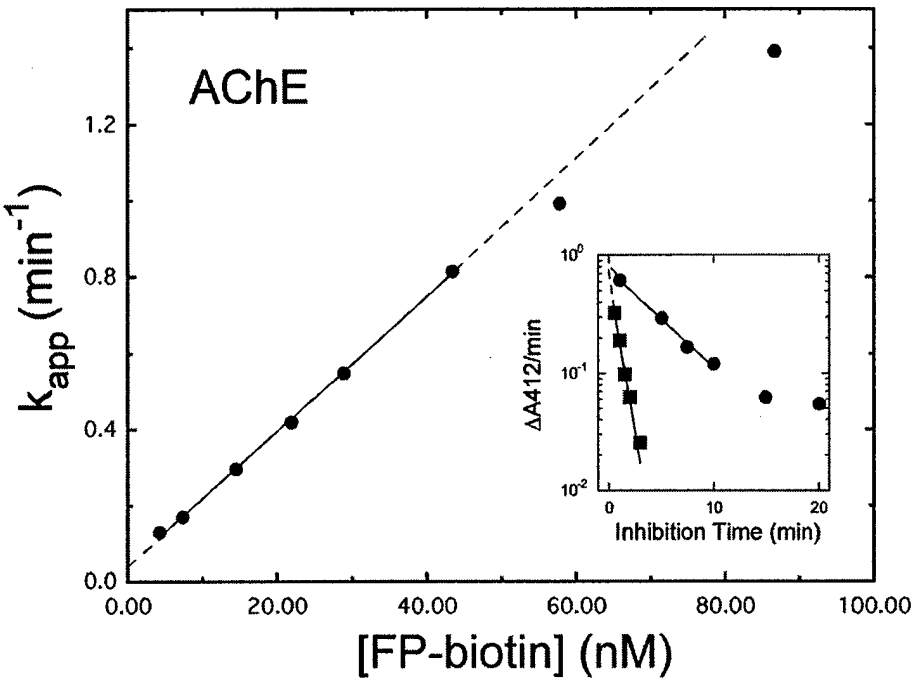

Figure 6.7. Rate constant for inhibition of human AChE by FP-biotin. k_{app} is the apparent first-order rate constant for the inhibition of AChE by FP-biotin. Points are the data; the solid line is from a linear fit, and the dashed line is an extrapolation of the fitted line. The slope yields the second-order rate constant, $k = 1.8 \pm 0.02 \times 10^7\ M^{-1}min^{-1}$.

Inset: Inhibition of AChE by 3.8 nM FP-biotin (circles) and 51 nM FP-biotin (squares). Solid lines are from fits to a first-order process. Data were fit using SigmaPlot. The slope yields k_{app} (min^{-1}) for a given FP-biotin concentration. The AChE concentration was 0.17 nM.

Reaction kinetics of purified human BChE with FP-biotin. Loss of BChE activity (0.35 nM BChE) was first-order for at least 90% of the reaction, at all concentrations of FP-biotin except for the very lowest (0.73 nM). See Figure 6.8 inset. A plot of the apparent first-order rate constant for the loss of BChE activity versus FP-biotin concentration was linear from 1.4 to 5.8 nM FP-biotin (Figure 6.8). Concentrations of FP-biotin were corrected for losses due to handling, and to 60% inhibition potency. The slope of this plot indicated a second-order rate constant for the reaction of human BChE with FP-biotin of $1.6 \pm 0.03 \times 10^8\ M^{-1}\ min^{-1}$.

The lower limit for the dissociation constant for FP-biotin and BChE was estimated to be greater than 5.8 nM. A more precise value could not be measured because the reaction was too fast to allow use of higher concentrations of FP-biotin.

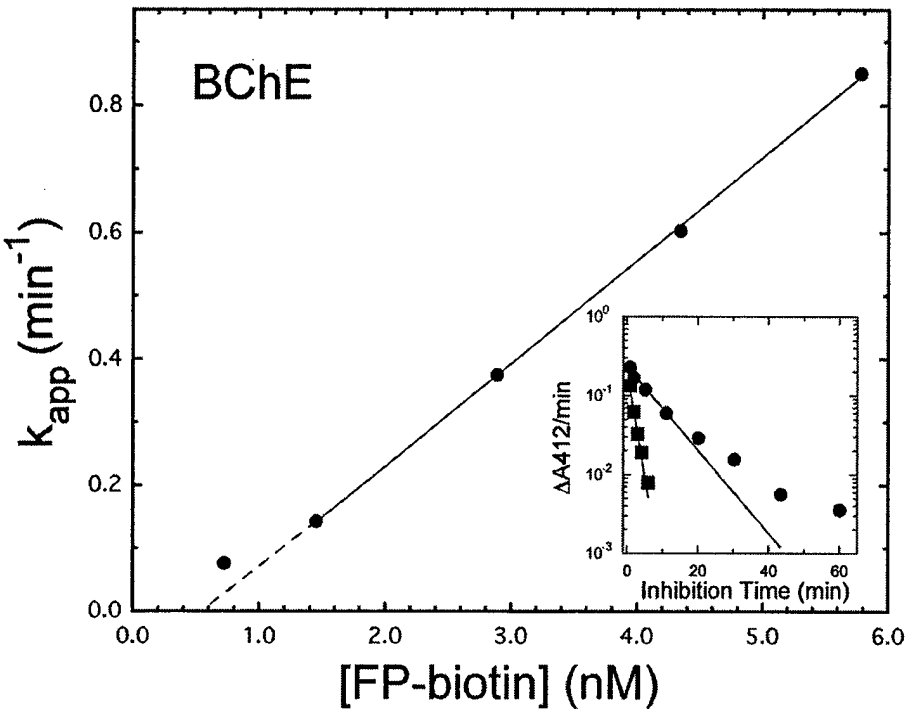

Figure 6.8. Inhibition of BChE by FP-biotin. k_{app} is the apparent first-order rate constant for the inhibition of human BChE by FP-biotin. Points are the data; the solid line is from a linear fit; the dashed line is an extrapolation of the fitted line. The slope yields the second-order rate constant, $k = 1.6 \pm 0.03 \times 10^8$ $M^{-1}min^{-1}$.

Inset: The inhibition of BChE by 0.73 nM FP-biotin (circles) and 5.8 nM FP-biotin (squares). Solid lines are from fits to a first-order process. Data were fit using SigmaPlot. The slope yields k_{app} (min^{-1}). The BChE concentration was 0.35 nM.

Discussion

The products of the reaction of AChE and BChE with FP-biotin are biotinylated AChE and biotinylated BChE where the biotin is covalently attached to the active site through the phosphate group. This reagent is suitable for the proposed task of identifying OP reactive proteins.

FP-biotin has good reactivity with human AChE and BChE despite its large biotin group. The spacer arm and biotin group are too large to fit inside the active site gorge. This proposition is supported by the fact that the biotin is available for reaction with avidin, a protein with a molecular weight of 68,000. We assume that the biotin either extends out the mouth of the gorge, or alternatively, extends through the putative backdoor (Gilson et al., 1994).

Stereoselective inhibition. The phosphate of FP-biotin is stereoisomeric (Figure 6.1). Our finding that only 60% of the FP-biotin had inhibitory potency is consistent with the interpretation that FP-biotin is a mixture of stereoisomers. The fraction of FP-biotin that failed to react with BChE also failed to label proteins in mouse brain, strongly suggesting that only one stereoisomer of FP-biotin is reactive.

Stereoselective inhibition by organophosphorus stereoisomers has been reported for BChE and AChE (Benschop et al., 1984; Hosea et al., 1995; Rodriguez et al., 1997; Millard et al., 1998; Ordentlich et al., 1999; Doorn et al., 2001). The stereoselective organophosphorus agents are soman, sarin, VX, isomalathion, malaoxon, and cycloheptyl methyl S-ethylphosphonyl thioate. The difference in reactivity between stereoisomers can be as high as 75,000 fold (Ordentlich et al., 1999).

Reactivity of FP-biotin. Comparison of the second-order rate constants of FP-biotin and other OP shows that FP-biotin is very potent (Table 6.1). The rate constant for FP-biotin reacting with BChE is similar to those for the nerve agents (cyclosarin and soman), for the pesticide metabolite diazoxon, and for DFP. Only chlorpyrifos oxon and MEPQ are significantly faster. Chlorpyrifos oxon is the active metabolite of the pesticide chlorpyrifos. MEPQ is related to VX in structure. Most of the active pesticide derivatives react more slowly. For AChE, only the nerve agents (cyclosarin, soman, and VX) and MEPQ react faster than FP-biotin. The reaction rates for sarin, chlorpyrifos oxon and FP-biotin with AChE are comparable, while the rates for the remaining representatives in Table 6.1 are slower.

Most of the OP in Table 6.1, including FP-biotin, react more slowly with AChE than with BChE. This can be explained by the finding of Millard et al. (Millard et al., 1999) that bulky OP molecules do not easily fit into the active site of AChE. The crystal structure of DFP-inhibited AChE showed that phosphorylation with DFP distorted the structure. The main chain loop that includes residues of the acyl-binding pocket moved almost 5 Angstroms. In contrast, BChE has more space near the active site and can accommodate large structures without bending out of shape (Nicolet et al., 2003).

Is FP-biotin expected to react with AChE in a living mouse? The second order rate constant for the reaction of AChE with FP-biotin can be used to estimate how long it will take the AChE in a mouse to react with FP-biotin. We have been injecting mice intraperitoneally with FP-biotin at a dose of 20 mg/kg. A mouse weighs 0.02 kg. If the FP-biotin is uniformly distributed in the mouse, this calculates to an FP-biotin concentration of 34 μM. Multiplying 34 μM by the second order rate constant (1.8×10^7 $M^{-1}min^{-1}$) yields a first-order rate constant of 612 min^{-1}. This means that half of the AChE should react with FP-biotin in 0.07 seconds. The second-order rate constant for BChE is faster than for AChE. Therefore it is expected that BChE in a mouse will also react with FP-biotin. We conclude that the rate constants are fast enough to expect AChE and BChE in a mouse to react with FP-biotin.

Table 6.1. Comparison of OP reaction rates with human AChE and BChE

OP	k, M^{-1} min^{-1} human AChE	k, M^{-1} min^{-1} human BChE	reference
FP-biotin	1.8×10^7	1.6×10^8	present work
cyclosarin	7.4×10^8	3.8×10^8	(Worek et al., 1998)
MEPQ	5.2×10^8	6.3×10^8	(Raveh et al., 1993)
soman	9.1×10^7	5.1×10^7	(Raveh et al., 1993)
soman PsCs	1.5×10^8	4.0×10^7	(Ordentlich et al., 1999)
VX	4.0×10^7	8.2×10^6	(Raveh et al., 1993)
sarin	1.8×10^7	1.2×10^7	(Raveh et al., 1993)
chlorpyrifos oxon	1.0×10^7	1.7×10^9	(Amitai et al., 1998)
tabun	4.0×10^6	4.7×10^6	(Raveh et al., 1993)
methyl paraoxon	1.6×10^6	4.2×10^4	(Skrinjaric-Spoljar et al., 1973)
paraoxon	7.0×10^5	1.9×10^6	(Amitai et al., 1998)
paraoxon		3×10^6	(Skrinjaric-Spoljar and Simeon 1993)
diazoxon	4.2×10^5	7.7×10^7	present work
dichlorvos	1.2×10^5	8.7×10^5	(Skrinjaric-Spoljar et al., 1973)
malaoxon	1.3×10^5	1.5×10^4	(Rodriguez et al., 1997)
malaoxon		1.1×10^4	(Main and Hastings, 1966)
DFP	5×10^4	1.7×10^7	(Millard et al., 1999)
oxydemeton methyl	2.2×10^4	2.2×10^3	(Worek et al., 1999)

Structures can be viewed at http://chem.sis.nlm.nih.gov/chemidplus/

FP-biotin, 10-(fluoroethoxyphosphinyl)-N-(biotinamidopentyl) decanamide
Cyclosarin, Cyclohexylmethylphonofluoridate
MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium
Soman, O-2-(3,3-dimethylbutyl)methylphosphonofluoridate
VX, O-ethyl-S-2(diisopropylamino)ethylmethyl phosphonothiate
Sarin, O-2-propylmethylphosphonofluoridate
Chlorpyrifos oxon, O,O-Diethyl O-3,5,6-trichloro-2-pyridyl phosphate
Tabun, O-ethyl-dimethylamidophosphorylcyanide
Methyl paraoxon, O,O-dimethyl-(4-nitrophenyl)-phosphate
Paraoxon, O,O-diethyl-(4-nitrophenyl)-phosphate
Diazoxon, O,O-diethyl-O-(2-isopropyl-6-methyl-4-pyrimidinyl)-phosphorothiolate
Dichlorvos, O,O-dimethyl-(2,2-dichloroethenyl)-phosphate
Malaoxon, O,O-dimethyl-S-(1,2decarbethoxy)-ethyl-phosphorothiolate
DFP, Diisopropylfluorophosphate
Oxydemeton methyl, O,O-dimethyl-S-(2-(ethylsulfinyl)ethyl)-phosphorothiolate

Key Research Accomplishments

- A new biomarker of OP exposure has been identified. This new biomarker is albumin.

- The reaction of human acetylcholinesterase and human butyrylcholinesterase with biotinylated OP was very fast, similar to the rate with sarin. This makes FP-biotin a suitable marker for OP reactive proteins. FP-biotin was used to identify albumin as a new biomarker of OP exposure.

Reportable Outcomes

- Manuscript to be submitted to Toxicological Sciences.
  Eric Peeples, Lawrence M. Schopfer, Ellen G. Duysen, Reggie Spaulding, Troy Voelker, Charles M. Thompson, Oksana Lockridge. Albumin, a new biomarker of OP exposure identified by mass spectroscopy.

- Manuscript to be submitted to Biochem J.
  Lawrence M. Schopfer, Troy Voelker, Cynthia F. Bartels, Charles M. Thompson, Oksana Lockridge. Reaction kinetics of biotinylated organophosphorus toxicant, FP-biotin, with human acetylcholinesterase and human butyrylcholinesterase.

- Accepted for publication 2004. Environ Toxicol Pharmacol.
  Oksana Lockridge, Ellen G. Duysen, Troy Voelker, Charles M. Thompson, Lawrence M. Schopfer. Life without acetylcholinesterase: the implications of cholinesterase inhibitor toxicity in AChE-knockout mice.

Conclusions

A new biomarker of exposure to organophosphorus poisons has been identified. This is a novel result that should be useful for diagnosing exposure.

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